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**MESENCHYMAL STEM CELLS AS ENDOGENOUS  
REGULATORS OF LEUKOCYTE RECRUITMENT;  
THE EFFECTS OF DIFFERENTIATION**

**By**  
**Hafsa Munir**

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Centre for Cardiovascular Sciences  
School of Clinical and Experimental Medicine  
College of Medical and Dental Sciences  
University of Birmingham  
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## **Abstract**

Mesenchymal stem cells (MSC) are a tissue-resident stromal cell population that are able to regulate immune responses, in particular the capacity for endothelial cells (EC) to support leukocyte recruitment. In this thesis we examined the ability of MSC from different sources (bone marrow, Wharton's jelly and trabecular bone) to regulate neutrophil recruitment to inflamed EC and how these responses are altered upon adipogenic differentiation of MSC. Using two flow based adhesion models with varying degrees of proximity between MSC and EC, we observed that all MSC populations suppressed neutrophil recruitment. IL-6 and TGF $\beta$  were identified as common bioactive agents found in all co-cultures. Upon differentiation, MSC exhibited a diminished capacity to suppress neutrophil, but not peripheral blood lymphocyte, recruitment. Loss of suppression by MSC-derived adipocytes was reversed by neutralising IL-6. Adipose tissue-derived mature adipocytes and culture differentiated pre-adipocytes did not recapitulate the effects of MSC-derived adipocytes. These data suggest that crosstalk between tissue-resident MSC and EC, dampens the endothelial response to cytokines and limits the aberrant infiltration of circulating leukocytes during inflammation. Upon adipogenic differentiation, MSC lose this regulatory capacity. This could impact on the beneficial effects of MSC in chronically inflamed sites where aberrant infiltration of leukocyte is a main driver of the disease.

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## List of abbreviations

AD	Adipocytes
ADMSC	Adipose tissue mesenchymal stem cells
ANOVA	Analysis of variance
BEC	Blood vascular endothelial cells
BMMSC	Bone marrow mesenchymal stem cells
BSA	Bovine serum albumin
C/EBP $\alpha$	CCAAT enhancer binding protein
C <sub>T</sub>	Threshold cycle
DC	Dendritic cells
EC	Endothelial cells
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ESAM	EC selective adhesion molecule
FABP4	Fatty acid-binding protein 4
FCS	Foetal calf serum
fMLP	N-Formylmethionine-leucyl-phenylalanine
HLA	Human leukocyte antigen
HSC	Haematopoietic stem cells
HUVEC	Human umbilical vein endothelial cells
IBMX	Isobutyl-1-methylxanthine
ICAM1	Intercellular adhesion molecule 1
IDO	Indoleamine 2, 3-dioxygenase
IFN $\gamma$	Interferon $\gamma$
IL-1 $\beta$	Interleukin-1 $\beta$
IL-6	Interleukin-6
IL-6ST	IL-6 signal transducer
ISCT	International Society for Cell Therapy
JAM	Junctional adhesion molecules
LFA1	Lymphocyte function-associated antigen 1
LG DMEM	Low glucose Dulbecco's Modified Eagle Medium
LPS	Lipopolysaccharide
MAC1	Macrophage-1 antigen
MCP1	Macrophage chemoattractant protein 1
MS	Multiple sclerosis
MSC	Mesenchymal stem cells
MSCGM	Mesenchymal stem cell growth media
NK	Natural killer cells
nmMSC	non-myogenic MSC
OA	Oleic acid
p	Passage
PBL	Peripheral blood lymphocytes

PBMC	Peripheral blood mononuclear cell
PBS	Phosphate Buffered Saline
PBSA	Phosphate Buffered Saline with bovine serum albumin
PD	Population doublings
PDGFR $\alpha$	Platelet-derived growth factor receptor $\alpha$
PDT	Population doubling time
PECAM1	Platelet/endothelial cell adhesion molecule-1
PGE <sub>2</sub>	Prostaglandin E <sub>2</sub>
PHA	Phytohaemagglutinin
PMN	Polymorphonuclear cells
PPAR $\gamma$	Peroxisome proliferator activated receptor $\gamma$
PSGL1	P-selectin glycoprotein ligand 1
RA	Rheumatoid arthritis
sIL-6R	soluble interleukin-6 receptor
SLE	Systemic lupus erythematosus
SOCS3	Suppressor of cytokine signalling 3
SS	Systemic sclerosis
STAT3	Signal transducers and activators of transcription protein 3
TBMSC	Trabecular bone mesenchymal stem cells
TGF $\beta$	Transforming growth factor $\beta$
TLR	Toll like receptor
TNF $\alpha$	Tumour necrosis factor $\alpha$
VCAM1	Vascular cell adhesion molecule 1
VLA4	Very late antigen 4
WJMSC	Wharton's Jelly mesenchymal stem cells

# **1. Introduction**



## **1.1. Overview**

This thesis primarily focuses on the capacity of mesenchymal stem cells (MSC) from different tissue sources to regulate leukocyte recruitment from flow, a key process in inflammation and how this capacity may change upon differentiation. This introduction will detail the process of leukocyte recruitment during inflammation, the phenotypic and immunomodulatory characteristics of MSC, cross-talk between MSC and endothelial cells (EC) and the ability of other stromal cells to regulate recruitment. It will further explore the role of ectopic fat in inflammatory diseases and whether MSC may contribute to ectopic fat in sites of chronic inflammation.

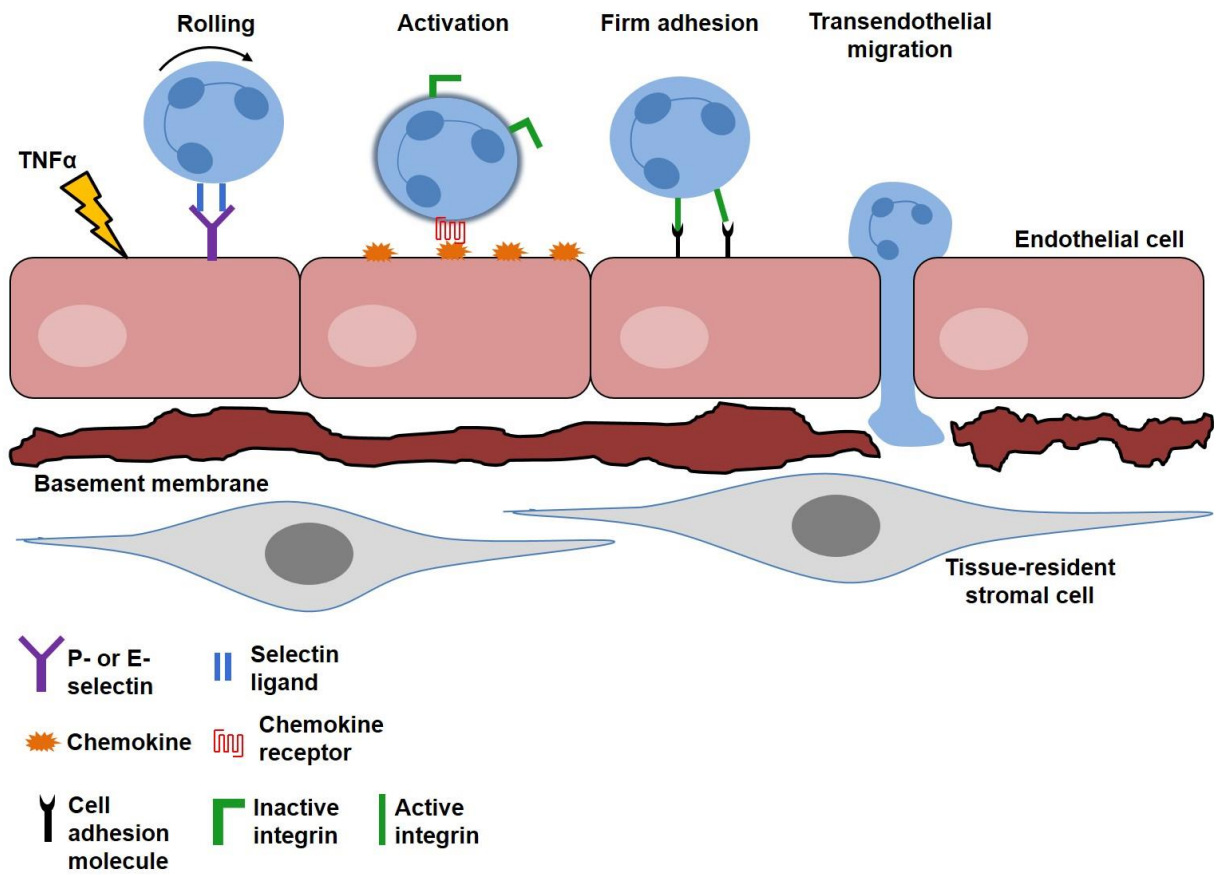
## **1.2. Inflammation**

Acute inflammation is characterised by localised pain, redness, heat and swelling that arise due to changes in endothelial behaviour and leukocyte recruitment within the tissue (Pober and Sessa, 2007). Initially, tissue-resident macrophages and mast cells become activated in response to pathogens or tissue damage and secrete pro-inflammatory cytokines and chemokines at the affected site (Eming *et al.*, 2007). These cytokines subsequently stimulate vascular EC to release nitric oxide and prostaglandins to cause vasodilation and increase local blood flow. This increases the number of leukocytes that come into contact with the endothelium and enables rapid recruitment of leukocytes to the inflamed tissue (Pober and Sessa, 2007). There is also increased vascular permeability causing plasma proteins and fluid to enter the tissue, which reduces the velocity of the blood and causes tissue oedema (Pober and Sessa, 2007).

Once at the site of inflammation, neutrophils bind to opsonised pathogens via their complement and immunoglobulin Fc receptors which leads to phagocytosis and eventual destruction of the pathogen (Eming *et al.*, 2007). Neutrophils generate reactive oxygen species and degranulate to release proteases that essentially kill the phagocytosed pathogen. As these molecules are highly toxic and can also damage host tissue, neutrophil activation must be tightly controlled. As such, during resolution neutrophils undergo apoptosis and are cleared by macrophages (Eming *et al.*, 2007). Failure to resolve inflammation can cause leukocytes to be continually recruited, leading to further tissue damage and eventually chronic inflammatory diseases such as rheumatoid arthritis (McGettrick *et al.*, 2012).

### **1.3. Leukocyte recruitment during inflammation**

Leukocyte recruitment is a critical step in the initiation of inflammation, a process which is tightly regulated by both EC and the tissue stroma. During inflammation leukocytes are recruited from the circulation into the afflicted site predominantly from postcapillary venules, where the haemodynamic shear stress is significantly lower than arteries ( $\leq 0.5\text{Pa}$  vs.  $1\text{-}2\text{Pa}$  respectively) (Lipowsky, 1985). EC respond to cytokines (tumour necrosis factor  $\alpha$ , TNF $\alpha$  and interleukin  $1\beta$ , IL- $1\beta$ ) produced by tissue-resident sentinel cells by increasing surface expression of cell-adhesion molecules and chemokines (Zhang *et al.*, 2011). These proteins promote leukocyte capture from flow and enable their migration into the inflamed site (summarised in Figure 1-1). Leukocyte extravasation is a multi-step process consisting of the following stages; margination, rolling, activation and firm adhesion, crawling and transendothelial migration [reviewed by (Ley *et al.*, 2007)].



**Figure 1-1: The multiple stages of the leukocyte recruitment cascade.**

### 1.3.1. Margination and capture

Leukocyte margination to the vessel wall arises as a result of erythrocytes traveling along the centre of the blood vessel, where the flow velocity is highest, causing leukocytes to be pushed to the vessel wall, where they come into contact with EC (Abbitt and Nash, 2003). Margination thus increases the frequency with which leukocytes come into contact with EC. Cytokine-stimulated EC express selectin molecules (P- and E- selectin) which mediate initial capture of neutrophils and lymphocytes from flow and cause them to roll along the vessel wall (Ley *et al.*, 2007; Luscinskas *et al.*, 1995). P-selectin is released from Weibel-Palade bodies fusing with the plasma membrane while E-selectin is synthesised *de novo* in response to cytokines (Ley *et al.*, 2007). Purified P-selectin and E-selectin were shown to be capable of capturing neutrophils from flow *in vitro*, indicating that neutrophil tethering is mediated by both P- and E-selectin (Abbitt and Nash, 2001; Lawrence *et al.*, 1994). In contrast, selectins were found to be less effective at capturing peripheral blood lymphocyte (PBL) from flow (Lalor and Nash, 1995). Indeed, blocking P-selectin did not completely abrogate rolling of CD4<sup>+</sup> T-cells on TNF $\alpha$ -stimulated EC and it was observed that interactions between vascular cell adhesion molecule 1 (VCAM1) and very late antigen 4 (VLA4) could support rolling (Luscinskas *et al.*, 1995).

P-selectin glycoprotein ligand 1 (PSGL1) is the most widely studied carbohydrate ligand that interacts with the N-terminal C-type lectin domain on P- and E-selectin (Ley *et al.*, 2007). Neutrophil rolling under high shear stress involves continuous binding and dissociation of selectins and their ligands (Sundd *et al.*, 2013). Coordinated rolling was recently found to be mediated by sling and tether interactions between P-selectin

and PSGL1. After margination, membrane tethers are formed at the rear of the neutrophil which bind to EC P-selectin via PSGL1 (Sundd *et al.*, 2010). These interactions are temporary and rapidly detach from the EC by pulling causing the tethers to roll over to the leading edge of the neutrophil (Sundd *et al.*, 2010). The tether forms a sling which then wraps around the neutrophil. The sling has punctate regions of PSGL1 along its length that interact with P-selectin on EC (Sundd *et al.*, 2012). The rapid formation and breaking of bonds between P-selectin and PSGL1 along the sling causes the neutrophil to slowly roll along the endothelium. Interestingly, tethers and slings express lymphocyte function-associated antigen 1 (LFA1) uniformly along their surface which interacts with intercellular adhesion molecule (ICAM) 1 and 2 on the EC surface (Sundd *et al.*, 2012). This interaction further reduces the velocity of the rolling neutrophils.

### 1.3.2. Activation and firm adhesion

The reduction in rolling velocity increases the exposure of neutrophils to chemokines presented on the endothelial surface which induce neutrophil activation. These chemokines can either be synthesised by the EC or actively transported from the adluminal surface (Ley *et al.*, 2007). At the luminal surface of EC, chemokines are bound to heparin sulphates to prevent shear forces of the blood detaching them from the cell surface (Massena *et al.*, 2010). CXC chemokines, such as IL-8, have been identified as key mediators of neutrophil activation and migration which signal via CXCR2 (Imaizumi *et al.*, 1997). Furthermore, inhibiting CXCR2, but not CXCR1, was previously shown to abrogate firm adhesion and migration of neutrophils through TNF $\alpha$ -stimulated EC (Luu *et al.*, 2000). In response to Interferon $\gamma$  (IFN $\gamma$ ) stimulation,

EC upregulated CXCL9, CXCL10 and CXCL11 which interacted with CXCR3 on T-cells (Sauty *et al.*, 2001). Binding of chemokines to corresponding receptors on the leukocyte surface induces activation of chemokine receptor associated G protein coupled receptors (Ley *et al.*, 2007). This triggers the activation of surface bound integrins which mediate firm adhesion.

Integrin-mediated interactions are slow forming, therefore rolling and chemokine activation of leukocytes are essential steps for ensuring integrin activation and formation of bonds. Integrins are homologous transmembrane cell adhesion molecules composed of an  $\alpha$  and  $\beta$  subunit which induce cell-cell adhesion and cell-extracellular matrix adhesion (via fibronectin and laminin) (Lefort *et al.*, 2012). Firm adhesion of neutrophils is primarily mediated by members of the  $\beta_2$  integrin family, LFA1. These integrins are highly expressed on neutrophils in an inactive form and must undergo a conformational change to become active and bind to their target ligands (cell adhesion molecules, e.g. ICAM1) on the endothelium (Takagi *et al.*, 2002). Chemokine-induced activation of integrins targets the cytoskeletal protein talin at the plasma membrane (Lefort *et al.*, 2012; Shamri *et al.*, 2005). Talin interacts with the cytoplasmic domain of the integrin  $\beta$  subunit and shifts it to an intermediate conformation (Lefort *et al.*, 2012). This further reduces the velocity of the rolling neutrophils. Binding of both talin1 and kindlin3 however, causes the integrin to change to a high affinity state. The integrins ( $\alpha_L\beta_2$ ) then form stronger interactions with their corresponding ligands (e.g. ICAM1) leading to neutrophil arrest (Lefort *et al.*, 2012; Wegener *et al.*, 2007). Chemokine activation of T-cells causes a conformational change in integrin VLA4 to a higher affinity state, enabling binding to VCAM1 expressed on EC through a similar mechanism (Ley *et al.*, 2007). This causes firm adhesion of T-cells to the EC surface.

### 1.3.3. *Crawling and transendothelial migration*

Firmly adherent neutrophils can then crawl along the endothelium to find the optimum site for transmigration. This is primarily mediated by integrin macrophage-1 antigen (Mac1) interactions with ICAM1 which ensure that the neutrophil remains attached to the EC while it protrudes pseudopodia to search for EC cell-cell junctions through which it preferentially migrates (Phillipson *et al.*, 2006). Arrested neutrophils can undergo migration through the endothelium after 1-2min and migration reaches a maximum by 10min (Luu *et al.*, 1999; Burton *et al.*, 2011).

The exact mechanism of neutrophil transmigration has not yet been fully elucidated. The process occurs mainly through a paracellular route (via intercellular EC junctions) and occasionally a transcellular route (through an EC) (Phillipson *et al.*, 2006). Intravital microscopy of murine cremasteric venules revealed that approximately 90% of neutrophils transmigrated through the paracellular route after IL-1 $\beta$  stimulation (Woodfin *et al.*, 2011). Adhesion molecules including platelet/endothelial cell adhesion molecule 1 (PECAM1), ICAM1 and 2, junctional adhesion molecules (JAM) A, B and C, CD99 (single-chain type-1 glycoprotein) and EC selective adhesion molecule (ESAM) expressed between EC junctions facilitate neutrophil and lymphocyte transmigration (Ley *et al.*, 2007; Woodfin *et al.*, 2011). Paracellular migration also requires dissociation of adherens junctions, namely those formed by endothelial VE-Cadherin (Su *et al.*, 2002).

In a murine model of ischemia-reperfusion injury, infused allogeneic bone marrow-derived leukocytes (deficient in ICAM2, JAMA, PECAM1 or TNF $\alpha$  receptors) showed differential capacity to migrate through the EC in cremasteric venules after IL-1 $\beta$  or TNF $\alpha$  treatment for 4h. Thus showing that these adhesion molecules facilitate transmigration in a cell-specific or stimulus-specific manner (Woodfin *et al.*, 2011). Specifically, PECAM1, ICAM2 and JAMA mediated neutrophil transmigration was induced in response to IL-1 $\beta$  stimulation but not TNF $\alpha$  (Woodfin *et al.*, 2009). TNF $\alpha$  was found to directly activate leukocytes migration via the TNF $\alpha$  receptor expressed on leukocytes and did not require the activity of ICAM2, JAMA and PECAM1 to exert its effect (Woodfin *et al.*, 2009). Prostaglandin D<sub>2</sub> has also been shown to be a promoter for neutrophil and T-cell migration although the exact mechanism of action is unknown (Tull *et al.*, 2009; Ahmed *et al.*, 2011).

Ligation of adhesion molecules (e.g. ICAM1) cause activation of diacyl glycerol and inositol-1, 4, 5- trisphosphate within EC inducing an increase in intracellular calcium levels (Woodfin *et al.*, 2011). This activates Rho GTPases which then stimulate myosin-light chain kinase, causing EC contraction. Consequently, adherens junctions between the EC open and the endothelial barrier weakens, allowing leukocytes to transmigrate (Woodfin *et al.*, 2011).

The phenotype of EC and their responses to cytokines are influenced by the local haemodynamic shear stress and the presence of stromal cells. Stromal cells such as MSC, fibroblasts and smooth muscle cells (Rainger and Nash, 2001; Luu *et al.*, 2013; McGettrick *et al.*, 2009) crosstalk with EC and regulate their capacity to induce



leukocyte recruitment from flow. Stromal cells may mediate their effects through the release of soluble chemokines which are presented to leukocytes on the surface of the EC (McGettrick *et al.*, 2010b). This has been shown to alter the recruitment of leukocytes to inflamed endothelium [reviewed by (McGettrick *et al.*, 2012)]. In this thesis we are primarily interested in MSC and their capacity to regulate recruitment and the mechanism underlying this process.

#### **1.4. Mesenchymal stem cell phenotype, origin and function**

Mesenchymal stem cells (MSC) are non-haematopoietic, multi-potent tissue-resident precursor cells that display multipotent differentiation potential and immunomodulatory capabilities (Pal *et al.*, 2009; Aggarwal and Pittenger, 2005; DiMarino *et al.*, 2013; Bianco, 2007). Initial studies showed that the BM contained a small population of stem cells of non-haematopoietic origin that exhibited colony forming capabilities, as well as, osteogenic, adipogenic and chondrogenic differentiation potential *in vitro* and *in vivo* (Friedenstein *et al.*, 1970; Friedenstein, 1976, 1974; Owen and Friedenstein, 1988). The bone marrow (BM) stroma was identified as the source of these cells as the isolated BM stem cells were able to rapidly adhere to culture plastics and displayed a fibroblastic morphology (Friedenstein *et al.*, 1970; Friedenstein, 1976, 1974). These cells were subsequently termed MSC and it was suggested that these cells were not specific to the BM and were in fact a common progenitor population residing in other mesodermal tissues that play a role in tissue homeostasis (Caplan, 1991; Pittenger, 1999). More recently it has been shown that MSC exist in small numbers in a variety of tissues including the bone marrow (BM), Wharton's jelly (WJ), adipose tissue (AD), dental pulp, brain, and spleen (Sotiropoulou *et al.*, 2006; Bianco, 2007). Within different

tissues, MSC are thought to exhibit heterogeneous phenotypes based on cellular size, colony-forming unit capabilities, surface marker expression, differentiation capacity, and function (Christodoulou *et al.*, 2013; Baksh *et al.*, 2007; Kern *et al.*, 2006). Indeed, growing evidence suggests that the MSC niche is unique in distinct tissues and that variation in tissue microenvironments may lead to tissue-specific differences in MSC functions (Magatti *et al.*, 2008; Wolbank *et al.*, 2007; Chen *et al.*, 2011; Chang *et al.*, 2006).

The primary function of tissue-resident MSC is believed to be tissue homeostasis and repair. Three mechanisms have been proposed to describe their contribution to these processes. Initially, based on their *in vitro* differentiation capacity MSC were thought to replenish cells by differentiating down specific lineages (Pal *et al.*, 2009). More recently, growth factors and cytokines secreted by MSC are thought to stimulate other cell populations to proliferate (Rubina *et al.*, 2009). For instance MSC secrete pro-angiogenic factors which stimulate EC and promotes new vessel formation, an important event in tissue repair (Rubina *et al.*, 2009). Alternatively, it has been suggested that MSC fuse with other tissue-resident cell types e.g. Purkinje cells or cardiomyocytes which then leads to *de novo* regeneration of damaged cells (Alvarez-Dolado *et al.*, 2003).

#### 1.4.1. Origin of MSC

MSC have been defined in the International Society for Cell Therapy (ISCT) 2006 guidelines (Dominici *et al.*, 2006) as a plastic adherent population that have colony-

forming capacity, trilineage differentiation potential (osteogenic, adipogenic and chondrogenic), express a panel of stromal markers (CD73, CD90, and CD105) and lack expression of leukocyte and haematopoietic markers (CD45, CD34, CD14 or CD11b, CD79a or CD19 and human leukocyte antigen-DR; HLA-DR). Additional surface proteins (e.g. CD146 and CD271) are thought to identify highly potent (based on clonogenic and immunosuppressive capacity) MSC subpopulations as assessed by their ability to suppress T-cell proliferation (Maijenburg *et al.*, 2012; Kuci *et al.*, 2010). However, no specific MSC marker - based on either surface expression or function - has been identified. Moreover, “MSC” markers are also found on non-MSC stromal populations (e.g. fibroblasts) indicating that this criterion is too generic for defining a specific population in tissue (Kundrotas, 2012; Sewell and Smith, 2011; Halfon *et al.*, 2011). Therefore, the MSC isolated from distinct tissue sources are likely to be heterogeneous populations of cells containing contaminating progenitor cells at various stages of lineage commitment. This may explain in part why there are differences in the differentiation capacity of MSC from distinct tissues. Also of concern is that the morphology, differentiation capacity, and expression of “MSC” markers are modified to varying degrees by *in vitro* culture conditions (Eggenhofer *et al.*, 2014). Identification of a unique, functionally relevant marker is urgently required to truly track MSC *in vivo* and elucidate their endogenous role in modulating inflammation. Further, this marker could also be used to track therapeutically infused MSC *in vivo* and assess their effector functions. Understanding the origin of MSC may identify early lineage-specific markers that are exclusively expressed on MSC and could be used to distinguish these cells from other stromal cells and to investigate their tissue distribution and any tissue-specific functions [reviewed by (Munir and McGettrick, 2015)].

Little is known about the developmental origin of MSC, however recent evidence suggests there are at least 2 distinct lineages: neural crest and mesoderm (Morikawa *et al.*, 2009; Crisan *et al.*, 2008). MSC can differentiate into cells of the neural lineages and subsets of murine BM-derived MSC have been reported to express neural crest stem cell-specific genes (Morikawa *et al.*, 2009), leading several groups to postulate this as their origin (Morikawa *et al.*, 2009; Nagoshi *et al.*, 2008). Additionally, murine neural crest-derived cells can migrate through the bloodstream to populate numerous tissues, including the BM; where they exhibit a differentiation capacity indicative of stem cells (Nagoshi *et al.*, 2008). In contrast, lineage tracing studies showed that cells from the primary vascular plexus give rise to perivascular cells that exhibit MSC-like properties (Crisan *et al.*, 2008; Feng *et al.*, 2011; Vodyanik *et al.*, 2010). Cells identified in tissue studies exhibited MSC defining markers or functional features (Jin *et al.*, 2013; Christodoulou *et al.*, 2013; Baksh *et al.*, 2007; Chen *et al.*, 2014). MSC in different tissues may originate from different sources which could lead to difference in their function (Nagoshi *et al.*, 2008; Crisan *et al.*, 2008; Feng *et al.*, 2011).

#### 1.4.2. MSC in the bone marrow niche

While MSC populations from different tissues exhibit some similar functional properties *in vitro* (Christodoulou *et al.*, 2013; Baksh *et al.*, 2007), it appears as though they may originally perform tissue-specific functions. For instance, in the BM, MSC contribute to the haematopoietic stem cell (HSC) niche and are key regulators of haematopoiesis (Méndez-Ferrer *et al.*, 2010; Ahn *et al.*, 2010; Omatsu *et al.*, 2010) and trafficking of other BM-derived cells into the circulation (Sugiyama *et al.*, 2006; Chow *et al.*, 2011;

Méndez-Ferrer *et al.*, 2010). Depletion of MSC or MSC-like progenitors caused an increase in HSC mobilisation (Méndez-Ferrer *et al.*, 2010) and augmented the expression of early myeloid selector genes by HSC, reducing their overall number in the BM (Omatsu *et al.*, 2010). This indicates that the presence of MSC in the HSC niche is essential for inducing HSC proliferation and maintenance in an undifferentiated state (Omatsu *et al.*, 2010). Indeed, stimulation of  $\beta$ -catenin in MSC has been shown to promote HSC self-renewal *in vivo* suggesting that this signalling pathway is involved (Ahn *et al.*, 2010).

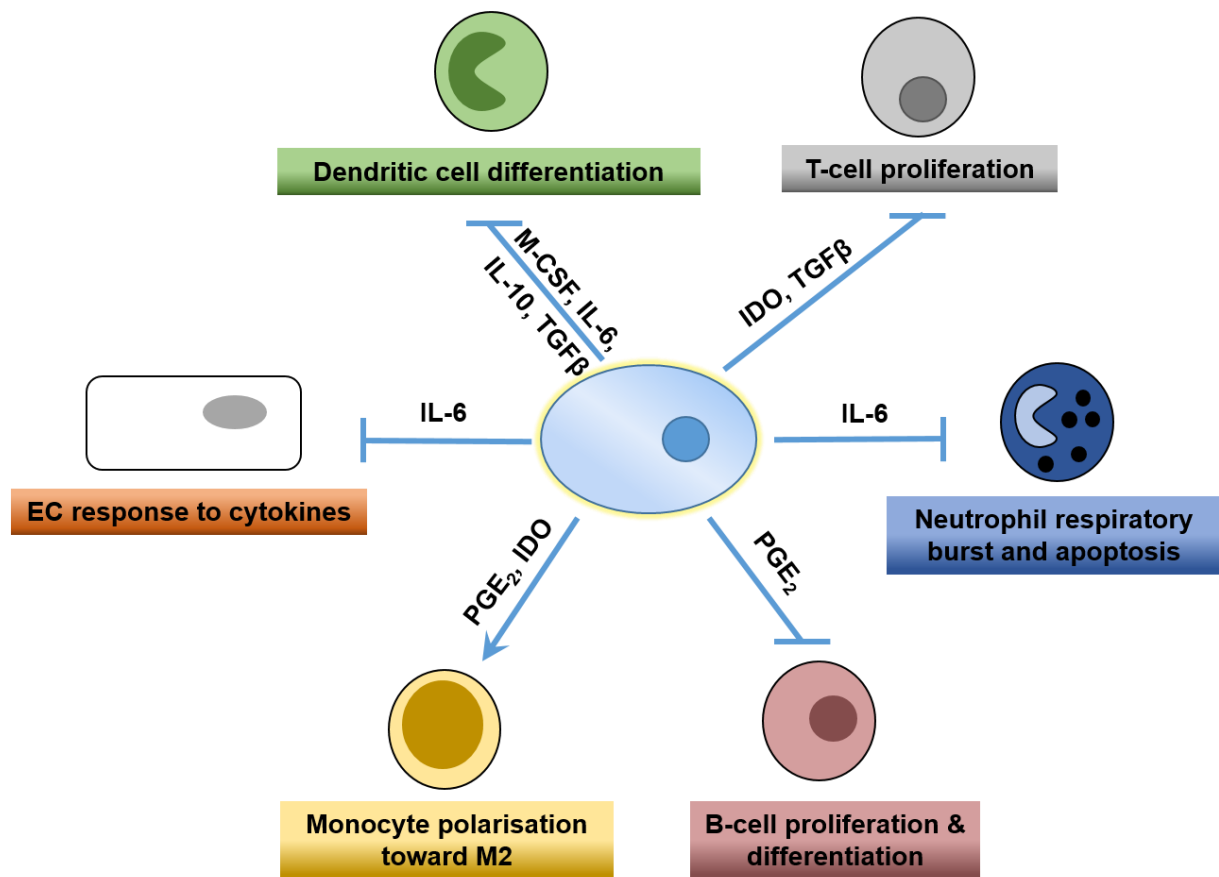
MSC can also “hold” HSC in the perivascular niche through CXCL12-CXCR4 dependent interactions, preventing them from exiting the BM into the bloodstream, akin to the mechanism previously reported for leukocytes (Méndez-Ferrer *et al.*, 2010; Sugiyama *et al.*, 2006; Beck *et al.*, 2014). Importantly, the expression of CXCL12 by MSC can be regulated by BM-resident CD169+ macrophages (Chow *et al.*, 2011). Depleting these BM macrophages reduced CXCL12 expression on MSC and in turn enhanced HSC egress into the bloodstream (Chow *et al.*, 2011). Thus, MSC play an integral role in maintaining HSC within the BM niche through soluble mediators, but also complex multi-cellular cross-talk with HSC and tissue-resident macrophages.

Evidence suggests that MSC may also regulate the trafficking of monocytes and B-cells from the bone marrow (Shi *et al.*, 2011; Chow *et al.*, 2011). During systemic infection, BMMSC up-regulated macrophage chemoattractant protein 1 (MCP1) in response to toll-like receptor (TLR) activation, promoting the egress of CCR2+ monocytes into the bloodstream (Shi *et al.*, 2011). This mobilisation of monocytes also

promoted HSC egress away from the stem cell niche (Shi *et al.*, 2011; Chow *et al.*, 2011) encouraging their maturation into leukocytes. This tightly regulated process required crosstalk between MSC, monocytes, and HSC to coordinate an appropriate immune response. BMMSC also down-regulated expression of CXCR4 by B-cells, which may have promoted their exit from the BM (Corcione *et al.*, 2006). Whether, MSC *in vivo* influence maturation of other leukocyte populations remains to be determined, although *in vitro* evidence suggests that they are capable of inhibiting differentiation of several leukocyte subsets (Djouad *et al.*, 2007; Ghannam *et al.*, 2010). A main function of tissue-resident BMMSC is to endogenously regulate the proliferation and maturation of HSC, and therefore indirectly influence leukocyte generation. Additionally, MSC may also regulate leukocyte egress in response to infection and/or inflammatory cues.

### **1.5. Immunomodulatory effects of MSC**

MSC also possess potent immunomodulatory capabilities and can regulate the survival, function and fate of different leukocyte populations (Figure 1-2) [reviewed by (Munir and McGettrick, 2015)]. MSC-mediated immunomodulation occurs through two mechanisms: release of soluble factors and cell-cell contact-dependent interactions. The majority of studies investigating the immunomodulatory capacity of MSC have been carried out on BMMSC (Le Blanc and Mougiakakos, 2012). However, recent evidence has shown that MSC from other tissues, namely clinical waste tissues such as dental pulp, umbilical cord and adipose tissue also exhibit immunomodulatory properties to varying degrees (Trivanović *et al.*, 2013).



**Figure 1-2: Immunomodulatory effects of MSC.** MSC alter the function and fate of several leukocyte populations through the secretion of soluble mediators. MSC can directly suppress neutrophil apoptosis and respiratory burst by reducing reactive oxygen species production. MSC suppress the proliferation and differentiation of PBL and dendritic cells and they can promote monocyte polarisation toward an anti-inflammatory (M2) phenotype. MSC can also modify the EC response to pro-inflammatory cytokines [taken from (Munir and McGettrick, 2015)].

These effects are mediated by soluble factors such as indoleamine 2, 3-dioxygenase (IDO), transforming growth factor beta (TGF $\beta$ ), interleukin-6 (IL-6) and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) (Trivanović *et al.*, 2013). Cell-cell contact with target immune cells has also been shown to play an important role in mediating several of the immunomodulatory effects, as discussed below. However, it is currently unknown whether these effects observed with culture expanded MSC represent their true endogenous function.

#### *1.5.1. Effects on innate immunity*

MSC are able to modulate the movement, activity, and survival of neutrophils. Conditioned medium from LPS-stimulated MSC promoted neutrophil chemotaxis and migration across blank filters. Furthermore, direct co-culture with early passage (p3-5) BMMSC was found to augment the activation of neutrophils (Brandau *et al.*, 2014). At p4, BMMSC inhibited apoptosis of resting and IL-8 stimulated neutrophils and dampened N-Formylmethionine-leucyl-phenylalanine (fMLP) induced respiratory burst in an IL-6 dependent manner (Raffaghello *et al.*, 2008). Lipopolysaccharide (LPS)-stimulation of tissue-resident glandular MSC enhanced their chemokine expression and conditioned medium from the MSC promoted neutrophil migration *in vitro*, in an IL-8 and macrophage migration inhibitory factor-dependent manner (Brandau *et al.*, 2010). Migrated neutrophils in the co-cultures became activated and had a significantly longer life span (Brandau *et al.*, 2010). This suggests that tissue-resident MSC may directly modulate the recruitment and activity of neutrophils responding to inflammatory insults or microbial challenge.



In another report, gastric cancer-derived MSC induced neutrophil activation via IL-6, downstream signal transducers and activators of transcription protein 3 (STAT3) and ERK1/2 signalling was also found to be involved. Activated neutrophils were further protected from spontaneous apoptosis (Zhu *et al.*, 2014). Similarly, umbilical cord WJMSC were found to suppress adult neutrophil apoptosis in a dose-dependent manner (Zhang *et al.*, 2015). Therefore, MSC from different tissue sources may promote neutrophil activation and survival in an IL-6 dependent manner. In certain contexts, direct cell-cell contact between MSC and neutrophils maximised the response (Zhu *et al.*, 2014). However, the reasons for this remain unknown and requires further investigations. In contrast, an *in vivo* study showed that intravenous infusion of BMMSC in a mouse sepsis model inhibited neutrophil migration into tissues and as a result limited multi-organ damage associated with sepsis (Németh *et al.*, 2009). *In vitro* experiments showed that LPS or TNF $\alpha$ -stimulation of BMMSC induced IL-10 production by macrophages which in turn reduced neutrophil recruitment (Németh *et al.*, 2009). Therefore, while MSC may dampen neutrophil recruitment, they could promote their survival and/or activation.

MSC are also capable of suppressing effector functions of natural killer (NK) cells (Le Blanc and Mougiakakos, 2012). Secretion of IFN $\gamma$  by IL-2 (Aggarwal and Pittenger, 2005; Spaggiari *et al.*, 2008) or IL-15 (Sotiropoulou *et al.*, 2006) stimulated NK cells was found to be suppressed by human BMMSC. BMMSC induced suppression was partially mediated by direct cell contact and PGE<sub>2</sub> (Spaggiari *et al.*, 2008; Sotiropoulou *et al.*, 2006). BMMSC also suppressed cytotoxic effects of cytokine-stimulated NK cells *in vitro* via the synergistic actions of IDO and PGE<sub>2</sub> (Spaggiari *et al.*, 2008). This

suggests that MSC can suppress the activity of several leukocyte populations using both soluble mediator and cell-cell contact dependent pathways.

MSC have also been shown to skew monocyte differentiation towards an anti-inflammatory M2 phenotype (Kim and Hematti, 2009; Maggini *et al.*, 2010). IDO and PGE<sub>2</sub> and direct cell-cell contact have also been implicated in BMMSC induced monocyte polarisation to IL-10 producing M2 macrophages (Kim and Hematti, 2009; Maggini *et al.*, 2010; François *et al.*, 2012). In a murine sepsis model, M2 macrophage-derived IL-10 reduced neutrophil infiltration *in vivo* following infusion of BMMSC (Németh *et al.*, 2009). In addition, human BMMSC suppressed differentiation of CD14<sup>+</sup> monocytes into dendritic cells *in vitro* (through GM-CSF, IL-4 and LPS) when cultured on opposite sides of a porous filter (Jiang *et al.*, 2005). Thus MSC may have the capacity to dampen inflammatory processes and promote resolution through its direct actions on several different leukocyte populations.

#### 1.5.2. Effects on adaptive immunity

The ability of murine and human MSC to modulate the proliferation and cytotoxic activity of T-cells has been extensively studied [reviewed by (Ma *et al.*, 2014)]. These assays are now widely used to test the potency of culture expanded MSC. MSC from BM, umbilical cord WJ and adipose tissue (AD) promoted T-cell survival *in vitro* and maintained them in a quiescent state by suppressing their proliferation (Ribeiro *et al.*, 2013; Selmani *et al.*, 2008). BMMSC also inhibited their production of pro-inflammatory cytokines to dampen their inflammatory effects (e.g. IFN $\gamma$ ) (Aggarwal and Pittenger, 2005). In this context, MSC-induced suppression was mediated by soluble factors (namely TGF $\beta$ , IDO and PGE<sub>2</sub>) (Li *et al.*, 2014a) and cell contact (Benvenuto *et al.*,

2007). These soluble factors acted synergistically to suppress T-cell proliferation and the effect was maximal when the MSC were in direct contact with target T-cells (Gu *et al.*, 2013). Additionally, BMMSC augmented the expansion of T<sub>reg</sub> cells in a HLA-G dependent manner which could be enhanced by addition of IL-10 (Selmani *et al.*, 2008). Therefore, as well as directly regulating the proliferation and activity of cytotoxic T-cells, MSC may also indirectly modify their effects by promoting the proliferation of local T<sub>reg</sub> populations.

In contrast, the ability of MSC to regulate other cell types of the adaptive immune system has not been clearly defined. Human BMMSC were found to preserve naive B-cells in a resting state, suppressing their proliferation, differentiation and antibody production (Corcione *et al.*, 2006). Murine BMMSC also inhibited proliferation of splenic B-cells from the follicular and marginal zone *in vitro* (Scheda *et al.*, 2010). Furthermore, direct contact between MSC and B-cells caused a reduction in the expression of chemokine receptors (CXCR5 and CCR7) on B-cells and dendritic cells (DC) (English *et al.*, 2008) which are essential for trafficking through lymphoid organs (Corcione *et al.*, 2006). Additionally, MSC may promote immunological tolerance *in vivo*, since co-culture with DC on opposite sides of a porous filter reduced expression of CD80/CD86 and HLA on the DC. This impaired their ability to stimulate T-cell expansion (Ramasamy *et al.*, 2007; Beyth *et al.*, 2005; Jiang *et al.*, 2005), although the mechanism(s) involved in mediating this effect were not demonstrated. The majority of effects on adaptive immune responses appear to be mediated by MSC-derived soluble factors. However, in certain contexts direct cell-cell contact has been shown to enhance the effect. The exact mechanism by which cell contact augments the suppressive effects of MSC on T-cells is unknown but may involve the PD-1

(programmed cell death protein 1) pathway (Schena *et al.*, 2010; Augello *et al.*, 2005). Indeed, blocking PD-1 in BMMSC co-cultures with T-cells reversed their suppressive effect on proliferation, indicating that this pathway plays a role in MSC-mediated suppression (Augello *et al.*, 2005). MSC can also indirectly modify immune cells through their interactions with EC to regulate leukocyte recruitment. As this is a major MSC function and the main focus of this thesis, it is discussed in detail in Section 1.7.2.

#### **1.6. Phenotypic and functional differences between MSC from different tissue sources**

MSC from adult and neonatal tissues fulfil the ISCT criteria, however differences in their proliferative capacity, differentiation potential and surface marker expression have been reported (Christodoulou *et al.*, 2013; Baksh *et al.*, 2007; Jin *et al.*, 2013; Kern *et al.*, 2006). Several studies revealed that neonatal MSC such as placenta, umbilical cord tissue or blood-derived MSC had a greater proliferative and clonal expansion potential than adult BMMSC and ADMSC (Li *et al.*, 2014b; Baksh *et al.*, 2007; Jin *et al.*, 2013; Christodoulou *et al.*, 2013). Neonatal MSC exhibited lower expression of senescence associated genes than adult MSC (Jin *et al.*, 2013; Li *et al.*, 2014b). WJMSC also displayed a greater osteogenic differentiation potential than BMMSC *in vitro* (Li *et al.*, 2014b; Baksh *et al.*, 2007) and adult ADMSC showed a greater proliferative and differentiation potential than BMMSC (Li *et al.*, 2014b).

Differences in the proliferation rate of distinct MSC populations reported in these studies could be due to differences in the levels of other stromal cells contaminating

the MSC culture. Within distinct tissues there may be different proportions of multipotent MSC to lineage committed progenitors (Post *et al.*, 2008). BMMSC cultures may contain more of these lineage committed stromal cell contaminants than umbilical cord-derived MSC. This would reflect differences in tissue composition at distinct anatomical sites and the differences in their proliferative and differentiation capacities.

In addition to phenotypic differences, MSC from several anatomical sites were found to modulate immune cell function to varying degrees (Li *et al.*, 2014b). WJMSC exerted a greater suppression on Phytohaemagglutinin (PHA)-induced T-cell proliferation than placental-derived MSC, BMMSC and ADMSC during co-culture (Li *et al.*, 2014b). A previous study also showed that BMMSC exhibited less potent suppressive effects than placental-derived MSC *in vitro*, in a similar model (Chang *et al.*, 2006). Additionally, co-culture of human umbilical cord blood-derived MSC with rodent macrophages significantly reduced secretion of pro-inflammatory cytokines (IL-1 $\alpha$ , IL-8 and IL-6) to a greater extent than BMMSC and ADMSC (Jin *et al.*, 2013). While BMMSC have a weaker suppressive effect than neonatal MSC populations, they have been shown to inhibit lymphocyte proliferation to a similar level as ADMSC (Roemeling-van Rhijn *et al.*, 2013; Puissant *et al.*, 2005). A conflicting report however showed that three distinct neonatal MSC populations (from the amnion, placenta and WJ) exerted similar levels of suppression on proliferation of alloreactive T-cells as BMMSC (Manochantr *et al.*, 2013). Discrepancies between studies could be due to differences in the methodologies used to isolate and culture the MSC. Therefore, while MSC from different sources mediate the same suppressive effects on different leukocytes subsets, the degree to which they mediate these effects and the mechanism by which they exert their effects may vary.

Collectively, the above suggests that MSC from neonatal tissues may be more potent than adult BMMSC and ADMSC in terms of their proliferative and immunomodulatory capacity (on T-cell proliferation). One explanation for this could be that MSC from neonatal tissues have undergone fewer population doublings prior to isolation than adult MSC. Indeed, culture expanded adult MSC have been reported to go through fewer population doublings in culture than neonatal MSC before undergoing replicative senescence (Ribeiro *et al.*, 2013). A recent study showed that senescent BMMSC had a reduced capacity to suppress PHA-induced T-cell proliferation when compared to dividing BMMSC (Sepúlveda *et al.*, 2014). In a murine model of LPS-induced sepsis, senescent BMMSC failed to protect mice from sepsis-associated lethality (Sepúlveda *et al.*, 2014). Further work is needed to assess whether senescence is associated with a loss of MSC-mediated immunosuppression. It is also possible that functional differences between MSC populations are due to differences in developmental origin of different MSC populations, a phenomenon previously observed in different smooth muscle cell populations (Sinha *et al.*, 2014).

#### **1.7. MSC crosstalk with EC and other tissue-resident stromal cells during homeostasis and inflammation**

MSC from different tissue sources are able to suppress immune cell proliferation and behaviour. However, little is known about whether tissue-resident MSC can influence the function of other tissue-resident cells in their vicinity, such as EC and whether there are tissue-specific differences. MSC reside in the perivascular niche in close proximity with EC lining the vasculature (blood and lymphatic) and other tissue-resident (stromal)

cells. Comparatively speaking we understand little about the interactions of MSC with these populations and their functional consequences.

#### 1.7.1. MSC interactions with stromal cells

Interactions between MSC and other stromal cells has mainly focused on the reparative properties of both cell types. Recent studies suggest that MSC may interact with other mesenchymal stromal cells within tissues during repair and bone remodelling to enhance their reparative functions (Tang *et al.*, 2009; Abdel Aziz *et al.*, 2007; Semedo *et al.*, 2009; Wu *et al.*, 2015; Li *et al.*, 2008). BMMSC reportedly migrate towards damaged bone in response to TGF $\beta$  released from osteoclasts at bone resorption sites (Tang *et al.*, 2009). BMMSC were thought to differentiate into osteoblasts and then promote remodelling of the bone (Tang *et al.*, 2009). In rodent models of tissue damage, injection of BMMSC or BMMSC-conditioned medium reduced fibrosis of affected organs, namely the kidneys, heart, liver and skin (Abdel Aziz *et al.*, 2007; Semedo *et al.*, 2009; Wu *et al.*, 2015; Li *et al.*, 2008). Soluble factors generated by the MSC (after infusion or in the conditioned media) may stimulate tissue-resident stromal cells to differentiate into specific cell types required to replenish the tissue. While MSC may limit tissue fibrosis and promote repair, the direct interactions between MSC and other tissue-resident stromal cell populations has not been explored; therefore, little is known about this potential function of MSC. Endogenous Gil1+ MSC on the other hand were found to contribute to tissue fibrosis after organ damage *in vivo* suggesting that the source of MSC, the tissue context and the type of fibrosis influence the effect of MSC (Kramann *et al.*, 2015). Further research is needed

to determine whether MSC and stromal cells influence each other to regulate the tissue microenvironment and therefore the local response during inflammation.

#### *1.7.2. MSC interactions with EC*

The effects of MSC on the behaviour of EC can be considered in three contexts: angiogenesis, vascular permeability and leukocyte recruitment. Interactions between BMMSC and EC have been described in these contexts while the effects of other MSC populations (e.g. WJMSC) on EC function has not yet been explored.

##### *1.7.2.1. Regulation of angiogenesis*

Under resting conditions, human and rodent BMMSC have been reported to release factors (e.g. Vascular endothelial growth factor  $\alpha$  and platelet-derived growth factor-BB) known to enhance the proliferation and migration of EC (Rahbarghazi *et al.*, 2012; Chen *et al.*, 2008; Dhar *et al.*, 2010). The production of these agents indicates that MSC have the potential to promote angiogenesis. In a murine model of wound repair, BMMSC (injected intradermally) increased EC and macrophage numbers at the site of the wound (Chen *et al.*, 2008; Wu *et al.*, 2007). Furthermore, this appeared to be an MSC-specific effect as, proliferation and migration of both human and murine EC *in vitro* was increased in the presence of conditioned medium from BMMSC but not dermal fibroblasts (Chen *et al.*, 2008). These studies suggested that MSC promoted wound healing by inducing angiogenesis. Furthermore, the tube forming capacity of EC was also augmented by BMMSC conditioned medium, indicating that MSC secreted pro-angiogenic factors (Wu *et al.*, 2007). The capacity of MSC to mediate



tube-formation *in vitro* has been extensively studied [reviewed by (Nassiri and Rahbarghazi, 2014)]. Co-injection of MSC with B16 melanoma cells increased tumour size and vessel area *in vivo*, indicating that they may also promote tumour angiogenesis (Suzuki *et al.*, 2011). A conflicting study found that MSC suppressed network formation through production of reactive oxygen species when in direct contact with rat lung microvascular EC in a matrigel model (Otsu *et al.*, 2009). Of note, other key stimulators of angiogenesis, such as shear stress and oxygen tension, were not modelled in these studies. Collectively, these studies suggest that in response to tissue damage, MSC may mediate their reparative effects, at least in part, through crosstalk with EC, by promoting angiogenesis.

#### 1.7.2.2. *Regulation of blood vascular permeability*

Recent evidence suggests that perivascular MSC also communicate with EC to regulate vascular permeability and maintain vessel integrity in resting and acute inflammatory conditions (Pati *et al.*, 2011; Zhao *et al.*, 2009; Pati *et al.*, 2010; Zhao *et al.*, 2010). Co-culture with MSC increased the stability of junctional molecules (e.g. VE-Cadherin and  $\beta$ -catenin) by inhibiting their turnover at the plasma membrane of EC, reducing endothelial permeability to FITC-dextran (Pati *et al.*, 2010). This effect was reproduced when EC were treated with conditioned medium from EC and MSC co-cultures, but not mono-cultures, implicating soluble mediators as the main drivers (Pati *et al.*, 2010). In LPS driven infection, infusion of BMMSC reduced pulmonary microvessel permeability and increased endothelial barrier function *in vivo*, thus reducing murine lung vascular permeability (Zhao *et al.*, 2009). Similar observations were made using a rat model of haemorrhagic shock (Pati *et al.*, 2011), indicating that

therapeutic administration of MSC may have beneficial effects for individuals with severe vascular damage. Tightening of the endothelial barrier may also regulate leukocyte infiltration into tissues. This could have important implications in chronic inflammatory disorders where aberrant infiltration of leukocytes from the blood plays a key role in the progression of the disease.

#### 1.7.2.3. *MSC regulation of leukocyte recruitment to inflamed EC*

A small number of studies described below indicate that perivascular MSC communicate directly with neighbouring endothelium to indirectly regulate leukocyte recruitment during inflammation. However, none have questioned whether the capacity to regulate this process varies between MSC from different sources and therefore whether it's likely to be tissue-specific.

Intravenous infusion of murine BMMSC increased the number of circulating neutrophils, whilst simultaneously decreasing circulating monocytes in a murine model of sepsis (Németh *et al.*, 2009). This suggests that MSC may actively inhibit neutrophil recruitment while simultaneously promoting monocyte recruitment. Although, the increase in levels of circulating neutrophils may be due to an increase in neutrophil production from the BM. A subsequent study showed that pre-treating pulmonary EC with conditioned medium from human EC and BMMSC co-cultures reduced their ability to support adhesion and migration of U937 cells (monocytic leukaemia cell line) in response to TNF $\alpha$  *in vitro* (Pati *et al.*, 2011). The reduction in migration was caused by tightening of endothelial adherens junctions (VE-cadherin and  $\beta$ -catenin) and a

reduction in expression of the adhesion molecule ICAM1 and VCAM1 (Pati *et al.*, 2011). Furthermore, a recent report showed that in a murine model of ischemia-reperfusion injury, systemic infusion of murine BMMSC reduced neutrophil recruitment to the injured jejunum (Kavanagh *et al.*, 2015). Thus, MSC *in vitro* and *in vivo* showed the capacity to reduce leukocyte recruitment through their interactions with EC.

In our laboratory, we have shown using an *in vitro* multi-cellular flow-based adhesion assay that BMMSC communicate with neighbouring vascular EC to limit leukocyte recruitment induced by pro-inflammatory cytokines (TNF $\alpha$  and IL-1 $\beta$ ) (Luu *et al.*, 2013). Specifically, BMMSC co-cultured in direct contact with EC (mimicking systemic infusion of MSC) reduced the numbers of both neutrophils and PBL adhering to and migrating through EC in response to cytokines, after 24h co-culture (Luu *et al.*, 2013). A two-way conversation between BMMSC and EC was essential for mediating these effects since co-culture, but not MSC mono-culture, conditioned medium mimicked the effects of direct co-culture. This indicates that the effect was mediated by soluble factors generated only during the co-culture.

IL-6 secretion was significantly upregulated in BMMSC co-culture compared to EC or MSC mono-cultures (Luu *et al.*, 2013). Knockdown of IL-6 in BMMSC reduced IL-6 production in co-culture indicating that IL-6 was primarily generated by the MSC (Luu *et al.*, 2013). Knockdown or blocking of IL-6 or soluble IL-6 receptor (sIL-6R) reversed the suppressive effects of BMMSC on neutrophil adhesion and migration to TNF $\alpha$ -stimulated EC and PBL recruitment to TNF $\alpha$  and IFN $\gamma$ -stimulated EC (Luu *et al.*, 2013). Thus, IL-6 signalling through sIL-6R was essential for suppressing leukocyte

recruitment in co-culture. TGF $\beta$  was shown to increase IL-6 production by MSC in co-culture and neutralisation of TGF $\beta$  partially reversed the suppression on neutrophil adhesion and migration (Luu *et al.*, 2013). Therefore, IL-6 and TGF $\beta$  appear to be involved in MSC-mediated suppression of recruitment with TGF $\beta$  appearing to act upstream of IL-6 (Luu *et al.*, 2013).

We observed that expression of ICAM1, VCAM1 and E-selectin at the genomic level was unaffected by co-culture despite the reduction in leukocyte recruitment observed (Luu *et al.*, 2013). However, protein expression of ICAM1 on the surface of TNF $\alpha$ -stimulated EC was reduced in co-culture (Luu *et al.*, 2013). ICAM1 and VCAM1 protein expression was significantly reduced in co-culture after treatment of EC with TNF $\alpha$  and IFN $\gamma$  (Luu *et al.*, 2013). Co-culture with BMMSC also inhibited the secretion of chemokines (CXCL8 and CXCL10) responsible for stabilising leukocyte adhesion, and driving onward migration (Luu *et al.*, 2013). Therefore, the suppression of ICAM1 and VCAM1 and chemokine expression on EC may in part explain the reduction in leukocyte adhesion and migration observed during BMMSC co-culture.

BMMSC and EC were also co-cultured on opposite sides of a porous filter to model the crosstalk that might occur within a tissue (Luu *et al.*, 2013; Munir *et al.*, 2015; McGettrick *et al.*, 2009). Again, it was observed that BMMSC were able to suppress neutrophil and PBL recruitment from flow (Luu *et al.*, 2013). This may suggest that MSC are endogenous regulators of leukocyte recruitment that mediate their effects through interactions with vascular EC.

Several studies utilising *in vitro* flow-based adhesion models have previously shown that, in addition to MSC, stromal cells from a variety of tissues (e.g. fibroblasts and podocytes) can modify leukocyte recruitment induced by inflammatory cytokines (Kuravi *et al.*, 2014; Lally *et al.*, 2005; Luu *et al.*, 2013; McGettrick *et al.*, 2009). Collectively, the abovementioned studies of stromal cells showed that they use conserved mechanisms to regulate recruitment, involving IL-6 and TGF $\beta$  as regulators (Kuravi *et al.*, 2014; Luu *et al.*, 2013; McGettrick *et al.*, 2009). IL-6 in conjunction with TGF $\beta$  was found to suppress neutrophil and PBL adhesion and migration through EC co-cultures with BMMSC or dermal fibroblasts *in vitro* (Luu *et al.*, 2013; McGettrick *et al.*, 2009). Indeed, in murine models of inflammation, IL-6 and TGF $\beta$  have previously been shown to reduce the inflammatory infiltrate by suppressing leukocyte recruitment (Ulich *et al.*, 1991; Tilg *et al.*, 1997).

Studies so far suggest that MSC crosstalk with EC to reduce vascular permeability and inhibit the recruitment of leukocytes in response to pro-inflammatory cytokines. Therefore, MSC could be used to treat chronic inflammatory conditions as they would suppress the aberrant influx of leukocytes into the tissue. Studies to date have not addressed whether MSC from different tissue sources have the same capacity to mediate the same effects as BMMSC or whether it is a tissue-specific function. Indeed other stromal populations, including EC and fibroblasts, display distinct spatial identities (Aird, 2012) that govern their behaviour. This allows them to establish tissue-specific 'address-codes' that actively regulate the recruitment of leukocytes to inflamed sites [reviewed by (Parsonage *et al.*, 2005)]. Furthermore, the requirement for cell-cell

contact between MSC and EC also needs to be examined as it could impact their therapeutic potential. If MSC require contact or close proximity with EC to undergo crosstalk then direct injection into the tissue would be required. Alternatively, if MSC are able to exert their effects from a distance then systemic infusion could be used. These issues need to be addressed before MSC can be used therapeutically to suppress leukocyte infiltration in patients with chronic inflammation.

### **1.8. IL-6 signalling during inflammation**

As noted above, IL-6 is implicated in several immunomodulatory effects of MSC. IL-6 is an abundant pleiotropic cytokine that has context-specific functions during inflammation. IL-6 acts in a pro-inflammatory manner by inhibiting differentiation of CD4<sup>+</sup> T-cells into T<sub>reg</sub> cells, promoting monocyte recruitment and inhibiting apoptosis of T-cells [reviewed by (Scheller *et al.*, 2011)]. However, as described above, IL-6 may also mediate anti-inflammatory effects such as suppression of leukocyte recruitment during inflammation. The IL-6 receptor complex is composed of a type I transmembrane glycoprotein receptor (IL-6R; CD126) and a type I transmembrane signal transducer (IL-6ST; gp130) (Barnes *et al.*, 2011). Most cell types, including EC and fibroblasts do not express IL-6R on their surface and rely upon soluble IL-6R (sIL-6R) for *trans*-signalling in order to respond to IL-6 (Barnes *et al.*, 2011). sIL-6R is generated by two distinct mechanisms; proteolytic cleavage of IL-6R on the surface of cells that express it (e.g. neutrophils and macrophages and some T-cell subsets) by matrix metalloproteinases (ADAM 17 or 10) or through expression of an IL-6R splice variant that lacks the transmembrane and cytosolic domains (e.g. by neutrophils and macrophages) (Müllberg *et al.*, 1993; Horiuchi *et al.*, 1994; Lust *et al.*, 1992). The sIL-

6R binds to free IL-6 and the complex then interacts with IL-6ST, which is ubiquitously expressed on all cell types (Scheller *et al.*, 2011). This interaction induces the activation of the transcription factor STAT3 through which the majority of the effects of IL-6 are thought to be mediated (Barnes *et al.*, 2011).

IL-6 may influence the sequence of events in acute inflammation through effects involving neutrophils, monocytes and EC. At the site of inflammation, chemokines such as IL-8 induced activated neutrophils to shed IL-6R which then signalled through EC via IL-6ST (Scheller *et al.*, 2011; Barnes *et al.*, 2011). IL-6 *trans*-signalling then inhibited production of IL-8 but promoted secretion of MCP1, inducing a transition from neutrophil to monocyte recruitment. This is thought to be a key step in the inflammatory process as it may prevent further tissue damage by reactive oxygen species and proteases secreted by the infiltrating neutrophils (Jones, 2005; Kaplanski *et al.*, 2003; Hurst *et al.*, 2001; McLoughlin *et al.*, 2004). These studies show that IL-6 acts in a pro-inflammatory manner by shifting the inflammatory response from neutrophils to monocytes.

However, we have previously shown that IL-6 acts in an anti-inflammatory manner by suppressing lymphocyte adhesion to cytokine-stimulated EC (McGettrick *et al.*, 2009). In this context IL-6 may reduce the expression of adhesion molecules by EC, thus reducing their capacity to support recruitment. Indeed, we showed that EC co-culture with BMMSC where IL-6 secretion was increased, recruitment of neutrophil and PBL was suppressed (Luu *et al.*, 2013). The increase in expression of ICAM1 and VCAM1 on cytokine-stimulated EC was inhibited by BMMSC co-culture suggesting that IL-6

signalling through the EC reduced their expression (Luu *et al.*, 2013). Therefore, IL-6 can act in a pro- or anti-inflammatory manner depending on the cell type it acts on and the context (e.g. healthy vs. chronically inflamed tissue) (McGettrick *et al.*, 2009).

### **1.9. Effect of inflammatory environment on MSC phenotype and function**

Inflamed tissue is a complex microenvironment that contains an array of soluble factors (such as pro- and anti-inflammatory cytokines). These agents can modify the actions of tissue-resident leukocytes, EC, and mesenchymal stromal cells, physiologically to remove the inflammatory insult and promote resolution. It has been suggested that tissue-resident MSC also respond to changes in their local microenvironment during inflammation (Prasanna *et al.*, 2010; Waterman *et al.*, 2010). Additionally, the inflammatory milieu may alter the phenotype of MSC, and for instance, in chronic conditions this may modify their immunomodulatory capacity.

#### *1.9.1. MSC response to inflammatory mediators*

Cytokines found in the inflammatory microenvironment can alter the phenotype and function of MSC. Pre-treatment of BMMSC, WJMSC and ADMSC with TNF $\alpha$  and IFN $\gamma$  for 18h altered the expression of TLR on the MSC surface and increased secretion of cytokines (e.g. IL-6) and chemokines (e.g. IL-8, CCL5) compared to unstimulated MSC (Raicevic *et al.*, 2011). Murine BMMSC treated with IFN $\gamma$  combined with either TNF $\alpha$ , IL-1 $\alpha$  or IL-1 $\beta$  for 24h exhibited greater expression of adhesion molecules such as ICAM1, VCAM1 and chemokines e.g. CXCL9 compared to untreated MSC (Ren *et al.*, 2008, 2010). Interestingly, treatment with individual cytokines had no significant effect



(Ren *et al.*, 2008, 2010) suggesting that MSC-mediated responses are tightly regulated and require the presence of several key agents for their induction. The cytokine-stimulated increase in adhesion molecule expression observed might be important for facilitating contact between tissue-resident MSC and infiltrating leukocytes. Binding of leukocytes to the MSC surface could potentially enhance the capacity of the MSC to exert their immunosuppressive effects.

IFN $\gamma$  stimulation of BMMSC and WJMSC for 72h induced expression of the anti-inflammatory agent IDO relative to non-primed MSC (Prasanna *et al.*, 2010). IDO has previously been identified as a key mediator of human MSC-induced suppression of T-cell proliferation (Prasanna *et al.*, 2010). In several studies, cytokine-stimulation augmented the immunomodulatory effects of MSC compared to untreated MSC (Prasanna *et al.*, 2010; Deuse *et al.*, 2011; Yoo *et al.*, 2009). Pre-treatment of BMMSC and placental or umbilical cord-derived MSC with IFN $\gamma$  for 48h increased their suppressive effect on T-cell proliferation and mixed lymphocyte reaction compared to naïve MSC (Jones *et al.*, 2007; Prasanna *et al.*, 2010). Furthermore, TNF $\alpha$  priming of BMMSC and WJMSC (for 72h) was found to increase their ability to suppress PHA-induced IL-2 secretion by T-cells (Prasanna *et al.*, 2010). In these studies, priming MSC appeared to augment their anti-inflammatory effects. However, enhancing the effector functions of MSC also has the potential for damaging consequences. For instance, co-infusion of murine BMMSC which have been primed with TNF $\alpha$  and IFN $\gamma$  (for 12h) with a C26 colon cancer cell line significantly increased tumour growth when compared to injection with non-primed MSC (Liu *et al.*, 2011). This was taken to suggest that the pro-angiogenic properties of MSC had promoted tumour growth in this model. Collectively, the above studies suggest that MSC are able to respond to

cytokines in the inflammatory milieu, thus enhancing their effector functions, primarily their immunosuppressive capacity. However, cytokine priming is not essential for MSC-mediated suppression of T-cell proliferation and secretion of pro-inflammatory cytokines, as initial studies showed that non-primed MSC are potent regulators of immune cell function (François *et al.*, 2012; Maggini *et al.*, 2010; Raffaghello *et al.*, 2008; Kim and Hematti, 2009). In addition, it is uncertain whether the *in vitro* effects of cytokine priming MSC truly represent the responses of MSC to the inflammatory environment *in vivo*.

Recently studies have suggested that MSC are able to polarise towards a pro-inflammatory MSC1 phenotype or an anti-inflammatory MSC2 phenotype through the engagement of different TLR. Poly (I:C)-stimulated TLR3 activation on MSC was initially shown to enhance the immunosuppressive effects of BMMSC through the release of anti-inflammatory agents such as IDO and IL-10 (Waterman *et al.*, 2010). In contrast, LPS ligation of TLR4 abrogated BMMSC-mediated suppression of T-cell proliferation and enhanced the production of pro-inflammatory cytokines (e.g. TNF $\alpha$ ) and collagen deposition (Waterman *et al.*, 2010). This was supported by *in vivo* experiments which showed that systemic infusion of TLR3-primed MSC into mice with lung injury ameliorated symptoms, whilst infusion of TLR4-primed MSC exacerbated the lung injury when compared to naive MSC (Waterman *et al.*, 2010). However, in other reports, MSC infusion reduced lung oedema and leukocyte infiltration in murine models of sepsis where high levels of LPS (TLR4 ligand) were present (Tyndall and Pistoia, 2009; Zhao *et al.*, 2009).

Subsequent *in vitro* studies have also produced conflicting results where secretion of IDO or PGE<sub>2</sub> and T-cell proliferation were variously enhanced, reduced, or unchanged by TLR3 and 4-stimulated BMMSC (Opitz *et al.*, 2009; Liotta *et al.*, 2008; Raicevic *et al.*, 2011). As we have recently reviewed, discrepancies between studies may reflect differences in experimental conditions (treatment concentrations and duration), source of MSC and the numbers used in each assay (Munir and McGettrick, 2015). However, these studies strongly indicate that MSC are highly plastic cells that can act as inflammatory sensors that respond to changes in levels of cytokines, danger signals and bacterial components in the tissue microenvironment.

#### *1.9.2. The potential effects of a chronic inflammatory environment on MSC behaviour*

While MSC exert a number of acute responses to inflammatory agents, whether effects persist or change during chronic inflammation is currently uncertain. Other mesenchymal stromal cell populations, namely fibroblasts and secretory smooth muscle cells, have previously been shown to undergo a transformation from an immunosuppressive, regulatory state to a pro-inflammatory state in chronic inflammatory diseases (i.e. rheumatoid arthritis, RA and atherosclerosis respectively) [reviewed by (McGettrick *et al.*, 2012)]. Therefore, it is possible that MSC undergo phenotypic changes (possibly to a pro-inflammatory state) in response to chronic inflammation.

An *ex vivo* study showed that human BMMSC isolated from RA patients showed impaired capacity to support haematopoiesis when co-cultured with CD34<sup>+</sup>

haematopoietic progenitors (Papadaki *et al.*, 2002). Furthermore, MSC from patients with either systemic lupus erythematosus (SLE) or RA exhibited reduced proliferation capacity and telomere length compared to BMMSC taken from healthy donors (Kastrinaki *et al.*, 2008; Nie *et al.*, 2010). BMMSC isolated from patients with multiple sclerosis (MS) or systemic sclerosis (SS) on the other hand did not exhibit any differences in their proliferation and differentiation capacity compared to healthy controls (Mallam *et al.*, 2010; Papadaki *et al.*, 2005; Larghero *et al.*, 2008). Interestingly, MSC from patients with SLE, RA and SS maintained the capacity to suppress T-cell proliferation (Kastrinaki *et al.*, 2008; Nie *et al.*, 2010; Larghero *et al.*, 2008). BMMSC from healthy donors cultured in medium containing 20% synovial fluid from osteoarthritis patients, increased the expression of IL-6 and IDO genes compared to those cultured with synovial fluid from post-mortem donors without joint inflammation (Leijts *et al.*, 2012). Proteomic analysis of RA BMMSC revealed changes in expression of cell cycle regulators namely a decrease in cyclin-D (which regulates transition from G1 to S-phase) when compared to healthy age and gender-matched BMMSC (Kastrinaki *et al.*, 2008). During chronic inflammation, BMMSC appear to undergo rapid proliferation followed by premature senescence as shortening of telomeres was observed (Kastrinaki *et al.*, 2008). The implications of the above changes in the phenotype of MSC or their immunomodulatory actions in response to chronic inflammation are not fully understood. Furthermore, since these studies were all carried out using BMMSC, whether MSC from other sources respond to the inflammatory microenvironment in the same manner remains to be explored.

### **1.10. Effect of differentiation on MSC immunomodulation**

In response to injury, tissue-resident or therapeutically administered MSC respond to environmental cues and may undergo differentiation in order to replenish the cells lost (Tang *et al.*, 2009). Whether differentiation under these conditions influences their immunomodulatory capacity, is a matter of current research interest. From a therapeutic perspective, loss of MSC immunomodulation upon differentiation could limit the use of MSC in treatment of inflammatory disorders.

Recent studies have compared the capacities of undifferentiated MSC and MSC-derived osteoblasts or chondrocytes to suppress T-cell proliferation and function (Niemeyer *et al.*, 2007; Montespan *et al.*, 2014; Lohan *et al.*, 2014). Human BMMSC and ADMSC were found to have a similar osteogenic differentiation capacity and MSC-derived osteoblasts from both sources suppressed the proliferation of allogeneic T-cells to the same extent as matched undifferentiated MSC (Montespan *et al.*, 2014; Niemeyer *et al.*, 2007). BMMSC and ADMSC-derived osteoblasts also retained expression of HLA-G (a potent immunosuppressive molecule involved in suppression of T-cell proliferation) (Montespan *et al.*, 2014). Furthermore, MSC from both sources continued to lack expression of major histocompatibility complex class II molecules after osteogenic differentiation (Niemeyer *et al.*, 2007). Therefore, as well as retaining their immunosuppressive potential, MSC also maintained their immune privileged status after osteogenic differentiation.

In addition, chondrogenic differentiation of human BMMSC did not alter their capacity to suppress proliferation of collagen type II induced T-cells from peripheral blood and synovial fluid of RA patients (Lohan *et al.*, 2014). BMMSC and derived chondrocytes also suppressed activation of T-cells (as measured by CD69 and CD25 surface expression) and reduced secretion of pro-inflammatory cytokines including TNF $\alpha$  and IFN $\gamma$  while simultaneously inducing expression of the anti-inflammatory cytokines, IL-4 and IL-10 *in vitro* (Lohan *et al.*, 2014). These data suggest that osteogenic and chondrogenic differentiation of MSC *in vivo* in response to tissue damage may not alter their endogenous immunosuppressive potential. In contrast, a recent study showed that murine MSC-derived chondrocytes lost their ability to suppress mitogen induced T-cell proliferation *in vitro* and loss of suppression was associated with a decrease in nitric oxide and PGE<sub>2</sub> levels (Ryan *et al.*, 2014). Conflicting findings may reflect differences in the assays used and MSC isolated.

Importantly, only the effects on T-cell proliferation have been analysed in the studies to date. It may be the case that while this effect is unaltered by differentiation, other effects on immune responses such as leukocyte recruitment are influenced by differentiation. In addition, the effect of adipogenic differentiation of MSC has not been explored and is of particular interest in this thesis.

### **1.11. Role of ectopic fat in chronic inflammation**

Ectopic fat refers to lipid accumulated in non-adipose tissue, such as the pancreas, skeletal muscle and the liver (Liu *et al.*, 2014). Ectopic fat deposits are associated with

a number of disorders including Duchenne muscular dystrophy (Uezumi *et al.*, 2010), myocardial infarction (Goldfarb and Roth, 2009) and RA (Arend *et al.*, 2013; Clements *et al.*, 2009; Schweitzer *et al.*, 1993). Accumulation of ectopic fat has also been implicated in metabolic disorders, namely atherosclerosis, dyslipidaemia and diabetes mellitus type II (Goodpaster and Wolf, 2004; Liu *et al.*, 2014). Interestingly, patients with fatty liver and pancreas exhibited elevated levels of inflammatory cytokines, suggesting a link between ectopic fat and chronic inflammation (Haukeland *et al.*, 2006; Smits and van Geenen, 2011).

#### *1.11.1. Role of inflammatory cytokines in ectopic fat deposition*

The main function of adipose tissue-derived mature adipocytes is to accumulate triglycerides as an energy store [reviewed by (Goossens, 2008)]. Initially, ectopic fat formation was thought to be due to ineffective lipid storage by adipose tissue. Indeed, obese patients had enlarged, dysfunctional adipocytes that were unable to support storage of excess lipid. This increased the uptake of lipid by other non-adipose tissues and increased ectopic fat deposition at these sites (Goossens, 2008). However, this concept was challenged by observations that non-obese patients with metabolic disorders also showed ectopic fat deposition (Liu *et al.*, 2014; Lim and Meigs, 2014). Lipodystrophy is a disorder characterised by abnormal production and distribution of fat within the body (Garg and Agarwal, 2009). Mutations in genes involved in fat storage and differentiation of pre-adipocytes (such as peroxisome proliferator activated receptor, PPAR $\gamma$ ) and lipid synthesis (1-acylglycerol-3-phosphate-O-acyltransferase 2 and Berardinelli-Seip congenital lipodystrophy) in adipocytes contribute to lipodystrophy (Garg and Agarwal, 2009). Impaired capacity to store lipid, increases the

levels of circulating lipids and promotes uptake by non-adipose tissues. Lipodystrophy was associated with elevated levels of TNF $\alpha$  within the adipose tissue (Sevastianova *et al.*, 2008). Thus, ectopic fat deposition is caused by an impairment of adipocytes to effectively uptake and store lipid.

Treatment of 3T3-L1 pre-adipocytes (cell line) with the inflammatory cytokines IL-6 and TNF $\alpha$  and the acute phase protein, serum amyloid A during adipogenic differentiation *in vitro* inhibited their capacity to differentiate into mature adipocytes (Gustafson and Smith, 2006; Filippin-Monteiro *et al.*, 2012). This was caused by a reduction in the expression of adipogenic transcription factors, PPAR $\gamma$  and CCAAT enhancer binding protein (C/EBP $\alpha$ ), in pre-adipocytes (Gustafson and Smith, 2006; Filippin-Monteiro *et al.*, 2012). In addition, inflammation has previously been shown to stimulate lysis of adipocytes to increase levels of circulating free fatty acids which can then promote lipid accumulation in ectopic sites (Salles *et al.*, 2012). Indeed, knockout of TNF $\alpha$  in mice on a high fat diet significantly increased the mass of fat pads compared to wild-type mice. Importantly, knockout mice showed reduced accumulation of triglycerides in ectopic sites (liver and skeletal muscle) and lower levels of circulating adipokines (Salles *et al.*, 2012). These results suggest that pro-inflammatory cytokines impair lipid uptake and storage by adipose tissue, thus increasing storage at ectopic sites.

#### 1.11.2. *Potential contribution of MSC to ectopic fat formation*

The development of ectopic fat could also be the result of inappropriate adipogenic differentiation of tissue-resident MSC in affected tissues. In a murine model of



Duchenne muscular dystrophy (induced by double knockout of dystrophin and utrophin), non-myogenic MSC (nmMSC) isolated from skeletal muscle exhibited enhanced adipogenic and osteogenic differentiation potential *in vitro* compared to wild-type nmMSC (Sohn *et al.*, 2013, 2015). *In vivo*, culture expanded nmMSC were found to differentiate into fatty acid-binding protein positive (FABP4<sup>+</sup>) adipocytes 2 weeks after transplantation into the skeletal muscle of dystrophin and utrophin knockout mice (Sohn *et al.*, 2013). Therefore, ectopic fat observed in this model may be attributed to abnormal differentiation of tissue-resident nmMSC (Sohn *et al.*, 2013, 2015).

In a murine model of muscle degeneration (induced by glycerol injection), destabilisation of the muscle fibres was associated with an increase in the number of adipocytes. After 2 weeks there was a significant increase in fat deposits and fibrotic tissue in the injured muscle (Uezumi *et al.*, 2010). Platelet-derived growth factor receptor  $\alpha$  (PDGFR $\alpha$ ) expressing mesenchymal progenitor cells isolated from murine skeletal muscle interstitium were the only cell type within the muscle capable of undergoing adipogenic differentiation *in vitro* and *in vivo* (Uezumi *et al.*, 2010). Ectopic fat formation in this model was suggested to be due to adipogenic differentiation of PDGFR $\alpha$ <sup>+</sup> progenitor cells. However, as MSC are not the only cell type that expresses PDGFR $\alpha$ , it is also possible that other PDGFR $\alpha$  expressing cells may contribute to ectopic fat deposition in the glycerol-induced muscle degeneration model.

In a different model of muscle injury (induced by infusion of cardiotoxin) ectopic fat deposition was not observed indicating that fat accumulation was stimulus-specific (Uezumi *et al.*, 2010). Interestingly, transplantation of PDGFR $\alpha$ <sup>+</sup> cells from the glycerol-

induced model referred to above, into mice with cardiotoxin-induced injury did not undergo adipogenic differentiation. In contrast, PDGFR $\alpha$ <sup>+</sup> cells from cardiotoxin-induced injury injected into muscle with glycerol-induced injury differentiated into PPAR $\gamma$  and C/EBP $\alpha$  expressing adipocytes (Uezumi *et al.*, 2010). This suggests that adipogenic differentiation of PDGFR $\alpha$ <sup>+</sup> cells *in vivo* is dependent upon the tissue microenvironment. Co-culture of PDGFR $\alpha$ <sup>+</sup> cells with myofibres inhibited their adipogenic differentiation *in vitro*. Therefore, the loss of myofibres during muscle degeneration might promote ectopic adipocyte accumulation in the skeletal muscle (Uezumi *et al.*, 2010).

Collectively, the foregoing suggests that under certain (potentially inflammatory, e.g. RA) conditions, ectopic fat deposition may arise from MSC causing them to change their phenotype, losing their ability to suppress the inflammatory response and perhaps taking on a stimulatory state and perpetuating inflammation. This might occur during 'classic' differentiation e.g., into adipocytes, or conversion into a non-specific state in chronically insulted tissue. The effect of adipocyte differentiation on the capacity of MSC to regulate immune processes such as leukocyte recruitment has not been examined and is a topic of this thesis.

#### 1.11.3. *MSC in ectopic bone deposition*

As well as ectopic fat, aberrant bone formation or calcification has also been described in fibrodysplasia ossificans progressiva (Culbert *et al.*, 2014), the vasculature of chronic kidney disease (Mizobuchi *et al.*, 2009), and the adipose tissue after intra-

abdominal surgery (Zamolyi *et al.*, 2006). Ectopic bone deposition may also be due to abnormal differentiation of MSC, as direct injection of murine BMMSC into the infarcted heart of mice induced formation of encapsulated structures. These structures contained calcifications and ossifications within scar tissue at the infarct site (Breitbach *et al.*, 2007).

### **1.12. Hypothesis and aims**

MSC and other tissue-resident stromal cell populations are able to engage in crosstalk with vascular EC and regulate the recruitment of circulating neutrophils and lymphocytes in response to pro-inflammatory cytokines (Kuravi *et al.*, 2014; Luu *et al.*, 2013; Lally *et al.*, 2005; McGettrick *et al.*, 2009). In particular, BMMSC are able to suppress TNF $\alpha$  and IL-1 $\beta$ -mediated leukocyte recruitment. Recently it has been shown that MSC from different anatomical sites have varying capacity to regulate other immune responses (Li *et al.*, 2014b). Therefore, MSC from different tissues may exhibit different effects on leukocyte recruitment. Furthermore, recently it has been suggested that MSC may contribute to ectopic fat development, a key event in the progression of dyslipidaemia and other metabolic disorders (Uezumi *et al.*, 2010; Sohn *et al.*, 2015; Breitbach *et al.*, 2007) also associated with sites of chronic inflammation. Therefore, we hypothesise that (i) MSC can modify the endothelial response to cytokines and thus act as endogenous tissue-resident regulators of leukocyte recruitment; (ii) that this ability may differ between MSC from different tissue sources; (iii) tissue-specific differences in MSC behaviour may have an impact on their therapeutic potential and (iv) that adipogenic differentiation may play a role in the loss of control of leukocyte recruitment observed in chronic inflammation.

To test these hypotheses, the aims of this project were:

1. To develop methods to isolate MSC from different tissues, characterise their behaviour in culture and assess whether they fulfil the MSC criteria.
2. To compare the ability of MSC from different tissues to regulate the recruitment of neutrophils by inflamed EC; including the effects of passage, requirement for close proximity between MSC and EC and the release of soluble mediators.
3. To assess the effect of adipogenic differentiation on the ability of MSC to regulate leukocyte recruitment.

## **2. Materials and Methods**

## **2.1. Isolation and culture of human umbilical vein endothelial cells**

Human umbilical cords were collected from the Human Biomaterials Resource Centre (HBRC; University of Birmingham) from full term births after caesarean section, with informed consent. Human umbilical vein endothelial cells (HUVEC) were isolated as previously described (Cooke *et al.*, 1993; Munir *et al.*, 2015). Briefly, the umbilical vein was cannulated at each end and rinsed with Dulbecco's Phosphate Buffered Saline (PBS; Sigma-Aldrich, Poole, UK). The vein was then filled with 1mg/ml collagenase type Ia from *Clostridium histolyticum* and incubated at 37°C for 20min. The umbilical cord was subsequently massaged to ensure detachment of HUVEC. The vein was flushed out with PBS, the cell suspension was collected and centrifuged at 400g for 5min. Cells were resuspended in complete culture medium consisting of M199 basal medium (Gibco, Paisley, UK) supplemented with 20% foetal calf serum (FCS), 35µg/ml Gentamicin, 10ng/ml epidermal growth factor, 1µg/ml hydrocortisone (all from Sigma) and 2.5µg/ml Amphotericin B (Life Technologies, Paisley, UK) and seeded into T25 flasks (SLS, Nottingham, UK). HUVEC were used at passage 1 (p1) for all assays. Commercial primary blood microvascular EC (BEC; PromoCell, Heidelberg, Germany) were obtained at p0 and expanded by three passages in culture. BEC were cultured in medium supplied by PromoCell (EC Growth Medium MV, PromoCell) and seeded onto T25 tissue culture flasks (SLS) until they reached 100% confluence at which point the cells were passaged at a ratio of 1:3 as described in Section 2.4. BEC were used at p4 for all assays.

## **2.2. Isolation and culture of human MSC**

### *2.2.1. Culture of BMMSC*

Commercial primary human BMMSC (Lonza Ltd, Basel, Switzerland) were obtained at p2 and expanded by three passages in culture. BMMSC were cultured in MSC growth medium supplied by Lonza (MSCGM™ BulletKit, Lonza Ltd) on T75 tissue culture flasks (SLS) until they reached 70-80% confluence at which point the cells were passaged at a ratio of 1:3 as described in Section 2.4. BMMSC were used at p5 for all adhesion assays unless otherwise stated.

### *2.2.2. Isolation and culture of WJMSC*

WJMSC were isolated from umbilical cords (in accordance with ethics stipulated by the HBRC) as previously described (Munir *et al.*, 2015), by resecting the blood vessels and cutting the remaining tissue into 2-3mm<sup>3</sup> fragments. Two methods were used to isolate MSC. The fragments were placed in a T25 tissue culture flask for two weeks, after which they were removed and outgrowth cells were cultured. Alternatively, the fragments were suspended in an enzymatic cocktail of 1mg/ml Collagenase type II from *Clostridium histolyticum* and 1mg/ml Hyaluronidase in PBS (all from Sigma) and incubated at 37°C on a rotator for 3h or 5h. The viscous cell suspension obtained after digestion was diluted 1:5 in PBS and passed through a 70µm filter (BD Falcon, Oxford, UK). The suspension was centrifuged at 400g for 5min and the pellet was resuspended in complete culture medium composed of low glucose (LG) DMEM with stable L-glutamine or Ham's F12 DMEM (all from Biosera, ZI du Bousquet, France) supplemented with 10% FCS, 100U/ml penicillin and 100µg/ml streptomycin (all from Sigma) or MSCGM.

### 2.2.3. Isolation and culture of TBMSC

Trabecular bone explants were taken from osteoarthritis patients after joint replacement and kindly provided by Dr Andrew Filer, Dr Mark Pearson and Dr A Thomas from the Royal Orthopaedic Hospital (Birmingham). The bone fragments were dissected into smaller fragments and placed in T25 flasks with complete LG DMEM growth media and incubated at 37°C in 5% CO<sub>2</sub>. Once the TBMSC migrated away from the explants and adhered to the flask, the tissue fragments were removed. Alternatively, the explants were suspended in an enzymatic cocktail of 1mg/ml collagenase type II from *Clostridium histolyticum* for 3h at 37°C and then centrifuged at 400g for 5min. The cells were resuspended in complete LG DMEM media and seeded onto a tissue culture flask. Enzymatic digestion did not yield sufficient numbers of cells for culture, therefore explant culture was used to isolate TBMSC for all work described.

Both WJMSC and TBMSC were allowed to reach 70-80% confluence and were then passaged at a ratio of 1:3 (described in Section 2.4.). The cells were expanded and characterised based on surface marker expression and differentiation potential at p3. The cells were also used at p3 for all subsequent adhesion assays.

### 2.2.4. Differentiation of human MSC

Adipogenic and osteogenic differentiation was induced by culturing MSC in Adipogenic Induction or Osteogenic Differentiation Medium (Lonza) according to the manufacturer's instructions for 21 or 14 days respectively.



### **2.3. Isolation and culture of human pre-adipocytes and mature adipocytes**

Human subcutaneous adipose tissue was collected from the HBRC with informed consent from healthy patients that had undergone open abdominal surgery. The tissue was cut into small fragments and suspended in DMEM/F-12 medium (Biosera) supplemented with 25mM HEPES and 2mg/ml collagenase type II (all from Sigma) at 37°C for 1h on a rotator. The digest was passed through a 70µm pore filter and then centrifuged at 400g for 5min. Floating mature adipocytes were harvested, resuspended in DMEM/F-12 medium and placed in culture for 24h. Due to their short life in culture (McNelis *et al.*, 2013), mature adipocytes were used 48h after cell isolation for subsequent assays. Pelleted pre-adipocytes were resuspended in DMEM/F-12 media supplemented with 10% FCS, 100U/ml penicillin and 100µg/ml streptomycin (all from Sigma) and transferred to a T75 culture flask as previously described (McNelis *et al.*, 2013). Pre-adipocytes were used at p1 for all functional assays.

#### ***2.3.1. Adipogenic differentiation of human pre-adipocytes***

Pre-adipocytes were differentiated into mature adipocytes at p1 for some functional assays. The cells were detached from culture flasks (described in Section 2.4.) and seeded onto the outer surface of 0.4µm 6-well Transwell filters (BD Pharmingen, Cowley, UK) at a density of  $5 \times 10^5$  cells/filter in complete DMEM/F-12 medium. After 24h, the medium was replaced with adipogenic differentiation media composed of DMEM/F-12, 166nM recombinant insulin, 0.2nM triiodothyronine (T3), 50µg/ml 3-isobutyl-1-methylxanthine (IBMX), 33µM Biotin, 17µM pantothenic acid, 2µM Rosiglitazone (PPAR agonist), 0.01mg/ml transferrin and 100nM cortisol (all from

Sigma) for 5 days as previously described (McNelis *et al.*, 2013). The medium was then replaced with differentiation media without IBMX and Rosiglitazone for a further 16-23 days after which functional assays were performed.

## **2.4. Detachment of cells from tissue culture flasks**

Human EC, MSC and adipocytes were detached by aspirating cell culture medium from flasks and adding sufficient 0.02% Ethylenediaminetetraacetic acid (EDTA; Sigma) to coat the flask. EDTA was aspirated after 2min and a sufficient volume of 2.5mg/ml trypsin (Sigma) was added to coat the growth surface of the flask and the cells were incubated at 37°C until the cells began to detach from the surface. The flask was tapped to completely dislodge the cells and the trypsin was inactivated by adding 8ml complete culture medium (dependent on the cell type). The cell suspension was centrifuged at 400g for 5min and either added to culture flasks for further expansion or seeded for adhesion assays. For seeding in adhesion assays, after trypsinisation cells were resuspended in 1ml culture media and 20µl of suspension was transferred to a cellometer counting slide and counted using a cellometer Auto T4 cell counter (Nexcelom; Salisbury Green, UK). Cells were subsequently diluted to the desired concentration in cell culture medium and seeded.

### **2.4.1. Freezing and thawing MSC**

All MSC (p5 BMMSC, p3 WJMSC and TBMSC) were detached from T75 culture flasks after reaching 70-80% confluence as described above. After centrifugation, the supernatant was aspirated and the pellet was resuspended in 3ml ice-cold serum free

Cryo-SFM (PromoCell). 1ml aliquots of cell suspension were transferred to 1.5ml cryovials (Greiner Bio-One, Stonehouse, UK) and placed into freezing containers which were stored at -80°C overnight. Cryovials were then transferred to a liquid nitrogen Dewar for long term storage. Each aliquot contained enough cells to seed a T25.

Prior to thawing MSC, the appropriate cell culture medium was heated to 37°C. Cells were rapidly thawed by gently warming the cryovial between hands and then resuspending in 8ml warm culture medium. Cells were centrifuged at 400g for 5min and supernatant was aspirated. The pellet was resuspended in 5ml appropriate MSC culture medium and seeded onto a T25 flask.

## **2.5. Isolation of neutrophils and peripheral blood lymphocytes**

Venous blood was obtained from healthy donors and placed in tubes containing EDTA (anti-coagulant; Sarstedt, Nümbrecht, Germany) after informed consent. The blood was separated on a gradient of 2.5ml Histopaque 1077 layered over 2.5ml Histopaque 1119 (Sigma). Blood was layered onto the gradient and centrifuged at 1120g for 40min. Neutrophils were harvested at the interface between Histopaque 1077 and 1119 above the erythrocyte layer and washed twice in a solution of 0.15% bovine serum albumin in PBS containing 1mM calcium chloride and 0.5mM magnesium chloride (PBSA; Sigma) by centrifuging at 400g for 5min. Neutrophils were counted using either a cellometer Auto T4 cell counter or a Z<sup>TM</sup> Series Coulter counter (Beckman Coulter, High Wycombe, UK) and resuspended in PBSA at the desired concentration.

To isolate PBL, the whole blood layered onto a Histopaque gradient was centrifuged at 1120g for 30min. The peripheral blood mononuclear cell (PBMC) layer was harvested (from above the 1077 layer) and washed twice in PBSA by centrifuging at 400g for 5min. The PBMC were resuspended in 10ml PBSA and placed in a T25 flask and incubated at 37°C in 5% CO<sub>2</sub> for 30min to plate out monocytes and platelets. Non-adherent PBL were collected and centrifuged at 400g for 5min. The cells were resuspended in PBSA, counted (as described above) and the concentration was adjusted to 2x10<sup>6</sup> cells/ml.

## **2.6. Proliferation kinetics of MSC in different culture media**

WJMSC were detached from the flask using trypsin/EDTA, counted using a haemocytometer and then seeded at 1,300 cells/cm<sup>2</sup> in T75 flasks and cultured in MSCGM, LG DMEM or Ham's F12 DMEM complete culture media for 5 days. Parallel BMMSC cultures were set up in MSCGM medium as a control. Images were taken of the flask using an inverted phase contrast microscope (Olympus, South-End on Sea, UK) from 5 random fields after 5 days. The cell number in each field was counted and averaged. The number of population doublings (PD) and the doubling time (PDT) were calculated as previously described (Christodoulou *et al.*, 2013) using the equations: **PD = (1/log<sub>10</sub>2)×log<sub>10</sub>(N<sub>t</sub>/N<sub>0</sub>)** and **PDT = t × [log<sub>10</sub><sup>2</sup> (log<sub>10</sub> N<sub>t</sub> – log<sub>10</sub> N<sub>0</sub>)]**, where N<sub>0</sub> is the number of cells counted at time zero; N<sub>t</sub> is the number of cells at each time point; and t is the number of hours between each time point.

## **2.7. Flow cytometry**

### *2.7.1. Characterisation of MSC phenotype*

Cultured BMMSC, WJMSC and TBMSC were characterised based on the expression of the surface markers CD44, CD73, CD90, CD105, CD146, CD271, CD14, CD34 and CD45. Cells were detached from the flask using 0.02% EDTA and Accutase (Life Technologies, Paisley, UK) and centrifuged at 400g for 5min. MSC were washed in PBSA solution and transferred to polypropylene tubes (BD Bioscience, Oxford, UK). Cells were incubated with antibodies against CD44-APC, CD73-FITC, CD90-BV421, CD105-PerCP-Cy5.5, CD271-AF647 (all from BD Bioscience), CD146-AF647 (Biolegends; London, UK) and CD14-PE (Immunotools, Friesoythe, Germany) CD34-CD45-PE (BD Bioscience) diluted at 1:50 for 20min at 2-4°C. Control cells were stained with fluorophore- and isotype-matched control antibodies. MSC were washed twice in PBSA by centrifuging at 400g for 5min. Pellets were resuspended in 200µl PBSA and analysed on a Cyan ADP flow cytometer (Beckman Coulter Inc., Pasadena, USA) and at least 5,000 live events were acquired per sample. Summit 4.3 software was used to analyse the data offline (Beckman Coulter). Data was expressed as the percentage positive cells expressing each marker above the level of the isotype control.

### *2.7.2. Detection of intracellular IL-6 and TGFβ signalling by flow cytometry*

EC and BMMSC mono-cultures or co-cultures were detached from surfaces using EDTA and Accutase (Gibco; as described in Section 2.4. with Accutase replacing Trypsin) at different time points after seeding the EC (15min, 30min, 1h, 4h, 6h and 24h). Cells were centrifuged at 400g for 5min and resuspended in 100µl PBSA. According to the manufacturer's instructions, Fix buffer I (BD Bioscience) was pre-

heated to 37°C and added to the cell suspension at a 1:1 volume. Cells were incubated at 37°C for 10min and then centrifuged at 400g for 5min. The cells were then washed twice in PBSA and the supernatant was aspirated. The pellets were vortexed and the cells were permeabilised by slowly adding ice cold perm buffer III (BD Bioscience). EC and BMMSC were incubated on ice for 30min and then centrifuged at 400g for 5min. Cells were washed twice in PBSA and resuspended in 300µl PBSA. 5µl anti-Smad2/3 or 20µl anti-STAT3 antibody (BD Bioscience) were added to the cells and incubated at room temperature for 30min. Control cells were stained with fluorophore- and isotype-matched control antibodies. The anti-Smad2/3 antibody was validated by treating PBMC and EC with 10ng/ml recombinant TGFβ for 30min and staining for Smad2/3. To validate the anti-STAT3 antibody, polymorphonuclear leukocytes (PMN) and BMMSC were treated with 100ng/ml IL-6 for 15min and stained for STAT3.

## **2.8. Assessment of multilineage differentiation of MSC**

After inducing differentiation down the osteogenic or adipogenic lineages for 14 or 21 days respectively, the cells (cultured in 6-well plates) were fixed in 10% neutral buffered formalin (composed of 100ml 40% formaldehyde, 4g sodium phosphate monobasic monohydrate, 6.5g sodium phosphate dibasic anhydrous [all from Sigma] and made up to 1L with distilled water) for 30min at room temperature and washed in distilled water. For staining lipid droplets in MSC-derived adipocytes, the cells were treated with 60% isopropanol and stained with 0.3% Oil Red O (Sigma) dissolved in isopropanol for 30min. The cells were washed and counterstained with heamatoxylin solution (Sigma). For staining calcium deposits in MSC-derived osteoblasts, fixed cells were stained with Alizarin Red dye (Sigma) dissolved in distilled water for 45 minutes.

Cells were then washed. Images of differentiated MSC were taken using an EVOS FL Imaging System (Thermo Scientific, Loughborough, UK).

## **2.9. Flow-based adhesion assays**

### *2.9.1. Co-culture of HUVEC and MSC in channel slides*

HUVEC were detached from culture flasks using trypsin/EDTA and seeded into pre-coated microchannel slides (VI<sup>0.4</sup>, Ibidi GmbH, Martinsried, Germany) at a density that would generate a confluent monolayer as previously described (Luu *et al.*, 2013). After 24h adherent BMMSC, WJMSC and TBMSC were dissociated using trypsin/EDTA and labelled with 5µM Cell Tracker Green (Life Technologies) for 30min in serum free MSCGM at 37°C. MSC were centrifuged at 400g for 5min and then resuspended in MSCGM with 10% FCS for a further 30min at 37°C and then counted using the Cellometer and adjusted to  $1.5 \times 10^5$  cell/ml. 30µl of suspension was added to chosen channels (enough to fill the growth area of the channel) and incubated for 1h at 37°C in 5% CO<sub>2</sub> to allow the cells to adhere (Luu *et al.*, 2013; Munir *et al.*, 2015). Non-adherent MSC were washed off with MSCGM and the microchannel slide was cultured for 24h. EC alone or co-cultures were treated with 100, 10 or 1U/ml TNFα (R&D Systems, Abingdon, UK) for 4h. Phase contrast and fluorescent images were taken of 5 random fields at 10X magnification. The number of EC per field were counted in the phase contrast images and the number of adherent MSC were counted in the corresponding fluorescent images. The ratio of MSC:EC was then calculated.

### 2.9.2. Establishing HUVEC and MSC co-cultures on Transwell filter

MSC and EC were co-cultured on opposite side of a porous Transwell filter as previously described (Luu *et al.*, 2013; McGettrick *et al.*, 2009). In short, BMMSC, WJMSC and TBMSC or MSC-derived adipocytes were detached from culture flasks using trypsin/EDTA, counted and resuspended in MSCGM. MSC were seeded on the outer surface of a 6-well 0.4µm Transwell filter at a density of  $5 \times 10^5$  cells/filter (BD Pharmingen) and incubated at 37°C and 5% CO<sub>2</sub> for 1h. The filters were subsequently inverted and transferred to a 6-well tissue culture plate (VWR) and cultured in the 5ml MSCGM for a further 24 hours (McGettrick *et al.*, 2009). EC were detached and seeded onto the inner surface of the filter at a density that would yield a confluent monolayer within 24h (McGettrick *et al.*, 2010b). Parallel EC mono-cultures were also established. All filters were cultured in MSCGM for 24h prior to running the flow-adhesion assay. Culture supernatants were harvested and centrifuged at 800g for 10min to remove cell debris. The supernatant was taken and stored at -80°C. The cells were stimulated with 100U/ml TNFα for 4h (to assess neutrophil recruitment) or 100U/ml TNFα and 10ng/ml Interferonγ (IFNγ; Peprotech Inc., London, UK) for 24h (to assess PBL recruitment) in fresh culture medium.

Alternatively, co-cultures were established by seeding MSC onto the bottom of a 6-well plate for 24h and seeding EC on a filter which was placed in the well. Parallel endothelial mono-cultures and direct co-cultures were set up as controls. To investigate the bioactivity of culture conditioned media, fresh EC mono-cultures were seeded onto filters and after 1h were treated with conditioned media from 24h MSC or MSC-derived adipocyte mono-cultures or co-cultures for 24h. EC were then stimulated



with 100U/ml TNF $\alpha$  in the same media for a further 4h before performing the adhesion assay.

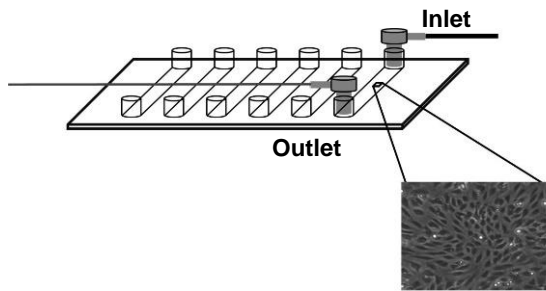
In some experiments, a neutralising antibody against either IL-6 (5 $\mu$ g/ml; clone 6708), transforming growth factor $\beta_1$  (10 $\mu$ g/ml; clone 9016) or a function blocking antibody against the IL-6R (5 $\mu$ g/ml; clone 17506; all from R&D Systems) was added when MSC or MSC-derived adipocyte co-cultures were established and was present for the duration of the co-culture and cytokine-stimulation.

### *2.9.3. The recruitment of leukocytes to EC from flow*

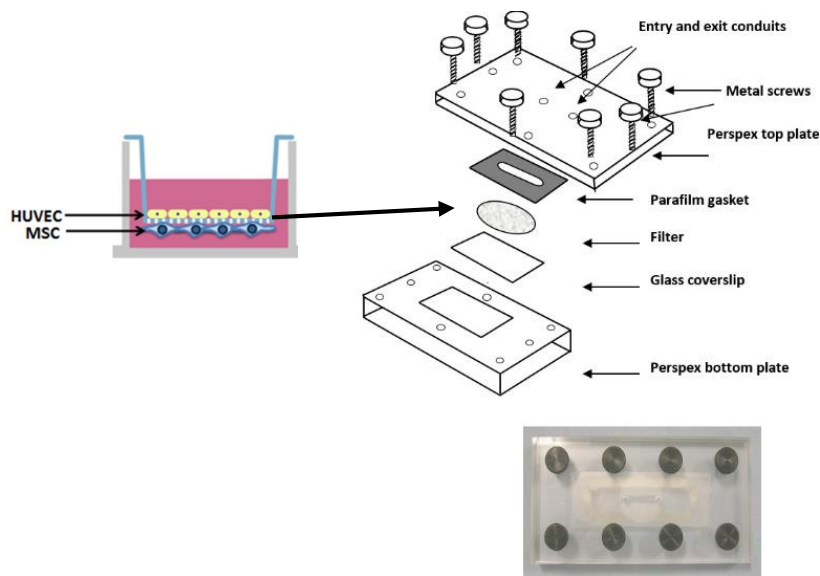
In the channel slide model, female Luer connectors were placed in the inlet and outlet holes of the microchannel slide creating a flow chamber (Figure 2-1A). In the filter model, EC mono-culture or co-culture filters were placed on a glass coverslip and cut out using a scalpel. The filter was then covered by a parafilm gasket that had a 20x4mm slot cut out over the filter region (Figure 2-1B).

The coverslip was positioned inside a custom made Perspex chamber consisting of two parallel plates. The top plate had an inlet and outlet hole cut out over the filter region (not covered by the plastic gasket) which created the flow channel over the EC monolayer (Figure 2-1B). Microchannel slides or filter chambers were connected to a perfusion system and placed on the stage of an inverted phase contrast/fluorescent video microscope in a Perspex chamber at 37°C.

### A. Microchannel slide model



### B. Filter model



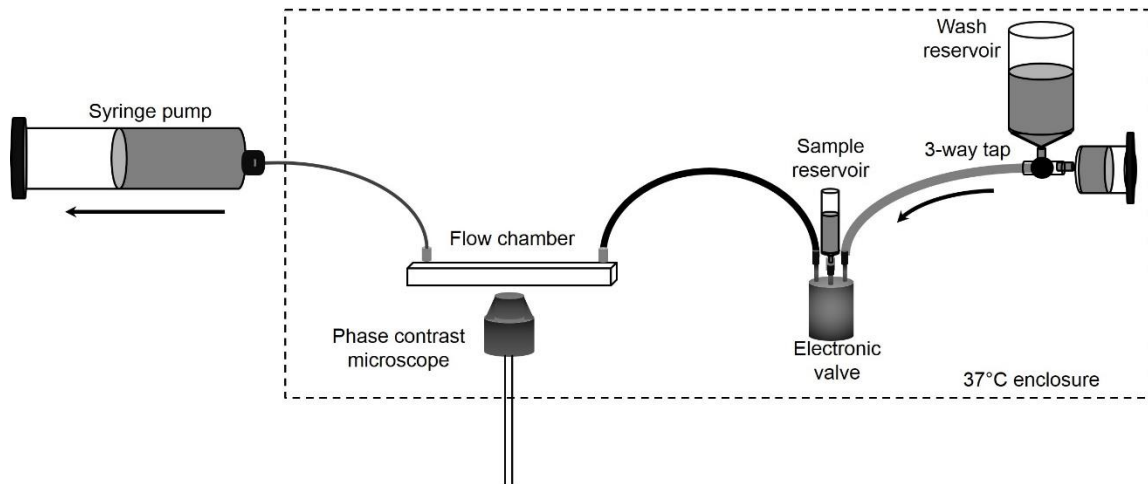
**Figure 2-1: Schematic of the microchannel slide and filter flow chambers.** (A) Schematic representation of the microchannel slide flow chamber indicating the growth area of the channel where the EC were seeded. The luer connectors that generated the inlet and the outlet port of the slide are shown. The dimensions of the channels were 0.44mm height, 17mm length and 3.8mm width. The growth area of the channel is 0.6 cm<sup>2</sup>. (B) Schematic representation of the filter flow chamber where EC and MSC were co-cultured on opposite sides of a Transwell filter. Image B was adapted from (McGettrick *et al.*, 2010a).

The perfusion system was composed of a “wash reservoir” containing PBSA and a “sample reservoir” containing leukocytes connected to a valve. An inlet tube coming from the microvalve was connected to the flow chamber enabling perfusion of PBSA or leukocytes over the EC (Figure 2-2). An outlet tube was connected to the flow chamber leading to a syringe pump which controlled the perfusion rate.

In the channel slide and filter models, neutrophils or PBL were diluted to a concentration  $1 \times 10^6$  or  $2 \times 10^6$  cells/ml respectively and perfused over the EC for 4min at a wall shear stress of either 0.05Pa (microchannel slides) or 0.1Pa (filters) as previously described (Luu *et al.*, 2013; Munir *et al.*, 2015; McGettrick *et al.*, 2009). This was followed by a 2min washout in cell free PBSA. 10 sec video recordings were made of 5 random fields along the centre of the flow chamber after 2min. PBSA was then flowed through the chamber for a further 5min and then 5 random fields were taken again after 9min post-perfusion.

#### *2.9.4. Analysis of leukocyte adhesion and behaviour*

Leukocyte behaviour was assessed using image analysis software (ImagePro 6.0, Data cell Ltd., Finchampstead, UK). At 2min and 9min post-perfusion, the number of adherent leukocytes/field were counted and classed as either rolling (phase-bright, sphere-shaped cell rolling along the endothelium much slower than free-flowing cells), stationary (typically exhibiting altered morphology and slowly migrating on the surface) or transmigrated (phase dark, spread cells that have migrated under the endothelium).



**Figure 2-2: Schematic of the perfusion system.** The microchannel slide or filter chamber was mounted onto the microscope stage and an inlet and outlet port was connected to either end of the channel. PBSA was flowed over the EC from the wash reservoir, connected to the electronic valve. The valve was turned on and a bolus of leukocytes were perfused over the EC for 4min. Then the valve was turned off and wash buffer was perfused for the remainder of the assay. The syringe pump was connected to the outlet of the flow chamber, regulating the flow rate of the perfused wash buffer or leukocyte suspension. Images were taken of 5 random fields using a phase contrast microscope. The image was adapted from (McGettrick *et al.*, 2010a).

The number of adherent leukocytes after 2min post-perfusion was averaged per field and converted to number/mm<sup>2</sup>/10<sup>6</sup> perfused based on the microscope field dimensions and the volume flow rate. Neutrophil behaviour was analysed at 9min post-perfusion and expressed as the percentage of adherent cells that were rolling, stationary or migrated.

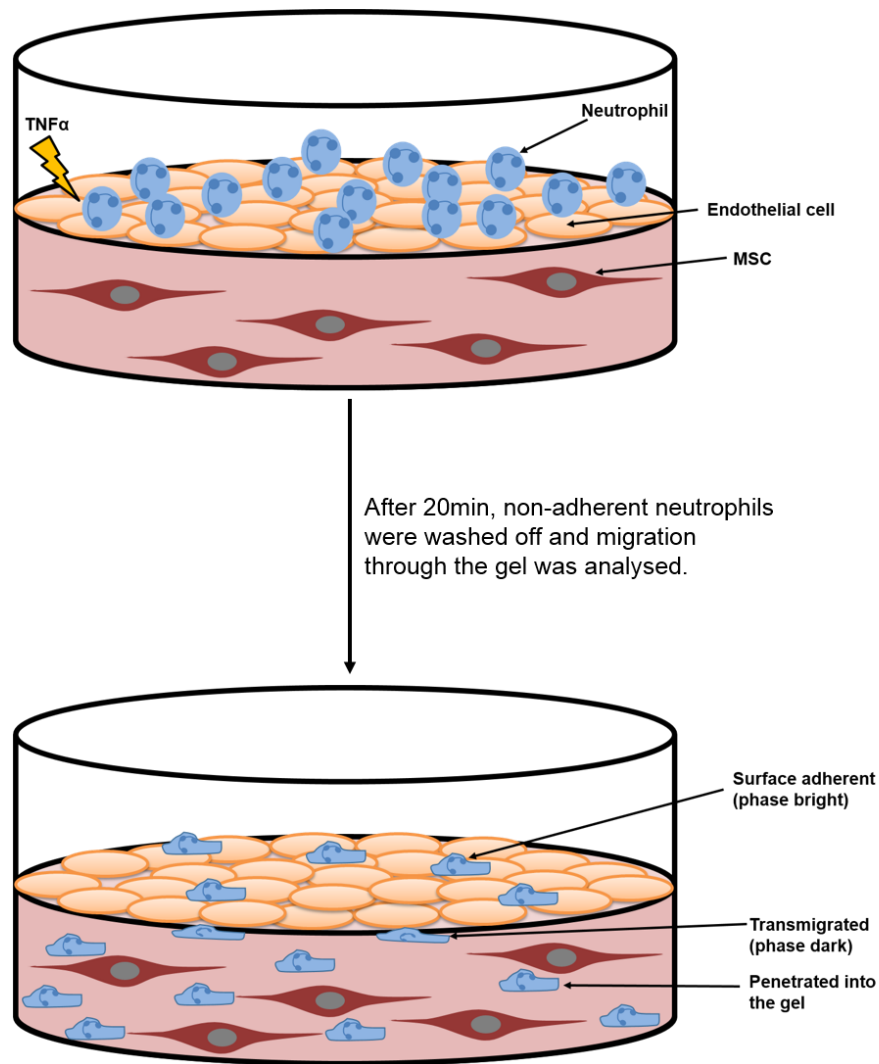
## **2.10. Leukocyte recruitment by 3D collagen gels incorporating EC and MSC**

BMMSC, WJMSC or BMMSC-derived adipocytes or osteoblasts were detached using trypsin/EDTA as described in Section 2.4. The cells were counted using a Cellometer and resuspended in an appropriate volume of ice cold FCS for seeding 25x10<sup>3</sup> cells/gel in 80µl. Collagen gels were generated by adding 3.32ml rat tail collagen (2.15mg/ml; First Link Ltd, West Midlands, UK) to a 10ml round bottomed tube (Appleton Woods Birmingham, UK). Subsequently, 680µl 10x concentrated M199 media (Sigma) was added to the tube and mixed by inversion. The pH of the gel was neutralised by slowly adding 1N NaOH (Sigma) until the colour changed from orange to pale pink as previously described (Jeffery *et al.*, 2013). All reagents and tubes were kept ice cold to delay the gels from setting.

To 8 wells of a 12-well plate 420µl collagen gel mixture was added. 80µl FCS (for EC mono-culture gels) or 80µl MSC or adipocyte or osteoblast cell suspension (for co-culture gels) was immediately added to each gel and mixed thoroughly to ensure even distribution of cells. The plate was incubated at 37°C and 5% CO<sub>2</sub> for 5-10min to allow the gels to set. 1ml MSCGM was added to gels and incubated at 37°C and 5% CO<sub>2</sub> for 24h. EC were detached from culture flasks using trypsin/EDTA and seeded on the gel

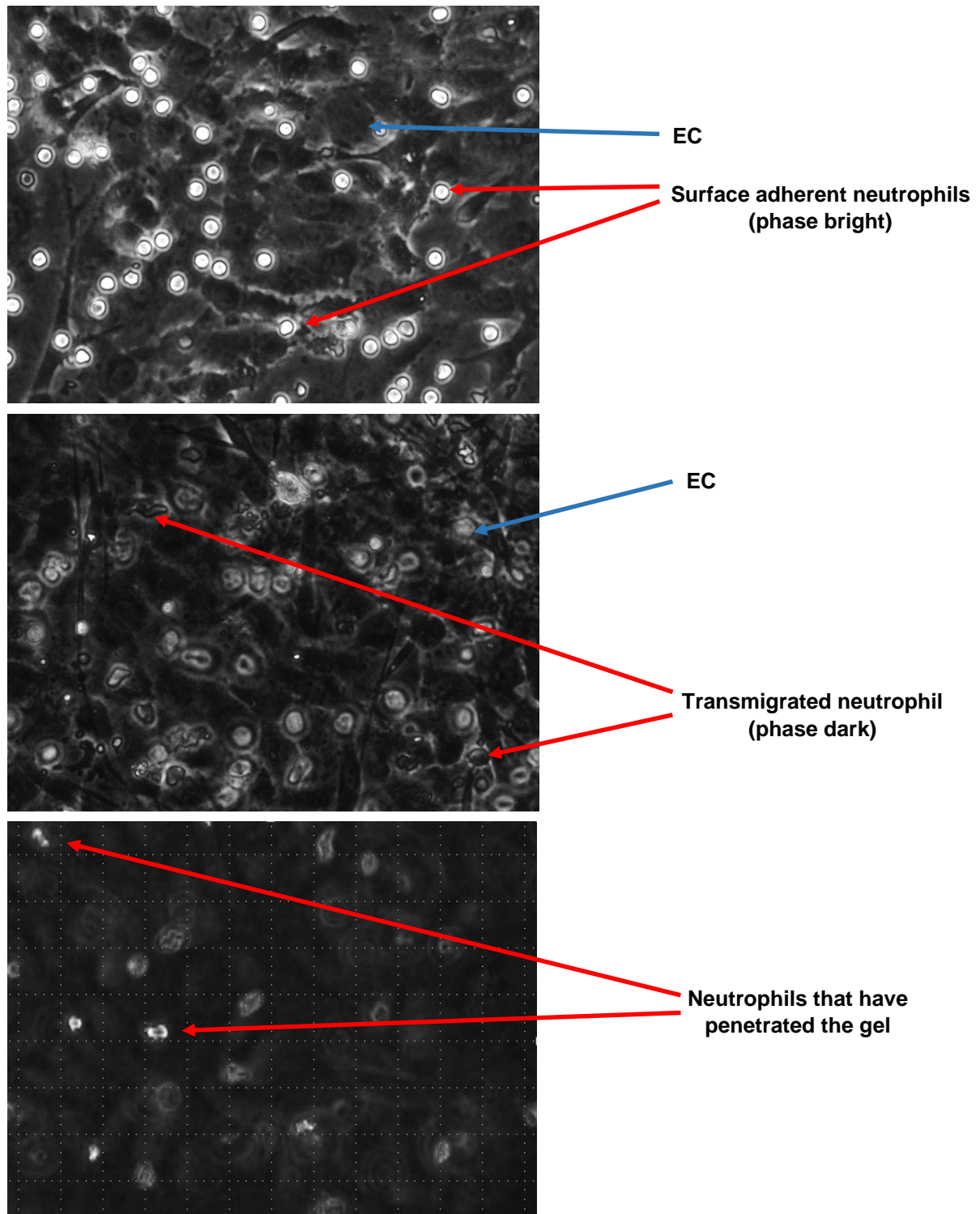
surface at a density that would yield a confluent monolayer within 24h. After 24h, gels were stimulated with TNF $\alpha$  for 4h to allow assessment of neutrophil migration (Figure 2-3).

Purified neutrophils were isolated as described in Section 2.5., counted and resuspended in PBSA at  $5 \times 10^5$  cells/ml (Figure 2-3). The gels were washed twice in PBSA and 1ml neutrophil suspension was added to each gel and incubated at 37°C and 5% CO<sub>2</sub> for 20min. Gels were washed three times in PBSA to remove non-adherent neutrophils. The plate was placed on the stage of an inverted phase contrast video microscope with a computer operated motorised focus in a Perspex chamber at 37°C. Digital z-stack images were taken at 2 $\mu$ m intervals through the thickness of the gel in 4 random fields using a digital camera controlled by ImagePro software. The gels were then placed back in the incubator for 2h. Then the gels were washed again, and Z-stack images were taken. In co-culture gels, z-stack images were also taken at 3 $\mu$ m intervals in 4 random fields to determine the location and number of MSC or adipocytes within the gel. The total number of neutrophils adherent on the EC surface and throughout the gel were counted on saved images using ImagePro software offline. Adherent neutrophils at the EC surface were labelled either surface adherent (phase bright) or transmigrated (phase dark; migrated just under the EC monolayer) (Figure 2-3 and 2-4). Using z-stack images at 20min and 2h, the numbers of neutrophils that penetrated the gel in each 50 $\mu$ m interval were also recorded (Figure 2-3 and 2-4). The neutrophil count was averaged over the 4 fields and converted to the total number/gel based on the known microscope field dimensions and the size of the well. The count was once again adjusted based on the field and well dimensions to determine the total MSC or adipocyte or osteoblast number/gel.



**Figure 2-3: Schematic of the 3D collagen gel model incorporating MSC and EC.**

A collagen gel based assay was setup to create a 3D stromal microenvironment in order to assess the effects of MSC and MSC-derived adipocytes and osteoblasts on neutrophil migration through EC and stroma. MSC or MSC-derived adipocytes and osteoblasts were seeded into a collagen gel matrix. After 24h, EC were seeded onto the gel surface. After a further 24h, gels were stimulated with  $TNF\alpha$  for 4h. Neutrophils ( $5 \times 10^5$ ) were added to the gels and incubated at  $37^\circ C$  and 5%  $CO_2$  for 20min. Non-adherent neutrophils were then washed off and neutrophil migration was assessed by taking z-stack imaging through the gel matrix. Cells were classified as surface adherent (phase bright on the EC surface), transmigrated (phase dark under the EC monolayer) or penetrated into the gel.



**Figure 2-4: Morphology of neutrophils and EC on the surface and within the 3D collagen gel matrix.** Representative images of HUVEC and neutrophils (surface adherent, transmigrated and gel penetrated) on collagen gel matrix after stimulation with TNF $\alpha$  for 4h. Images are representative of n=6 independent experiments. Images were taken at 20X magnification.



Total adhesion was expressed as a percentage of the total number of neutrophils added to each gel. Surface adherent, transmigrating and gel penetrated neutrophils were calculated as a percentage of the total adherent. The average depth of neutrophil penetration was calculated by multiplying the midpoint depth of each 50µm interval by the average number of neutrophils found each interval, adding the numbers for each interval, and then dividing this value by the total number of neutrophils that penetrated into the gel. as previously described (Jeffery *et al.*, 2013).

## **2.11. Quantification of protein in culture supernatants**

### **2.11.1. ELISA**

Sandwich enzyme-linked immunosorbent assay (ELISA) kits were used to quantify human IL-6, sIL-6R and CD105 levels (R&D Systems) in supernatants from microchannel slides and filters. Mono-cultures supernatants were harvested from 24h EC or MSC cultures on filters or microchannel slides while co-cultures supernatants were harvested after a further 24h culture. All supernatants were collected prior to cytokine stimulation. The kits were used according to the manufacturer's instructions. The absorbance was measured at 450nm and 540nm using a Bio-tek Synergy™ HT Multi-Detection Microplate Reader (Bio-Tek, Swindon, UK). The concentration of protein was calculated based on interpolation using a standard curve of calibrants supplied with the kits.

### *2.11.2. Membrane-based sandwich cytokine array*

A Human XL Cytokine Array Kit (R&D Systems) was used to screen for cytokines present in supernatants (same supernatants as tested in Section 2.11.1.) according to the manufacturer's instructions. A panel of 102 cytokines were analysed (Table 2-1). Data were analysed after 5min exposure of the membrane to X-ray film. The film was then scanned and the average pixel density (termed integrated density) for each analyte was quantified using ImageJ software. This analysis was semi-quantitative.

## **2.12. Gene expression analysis**

### *2.12.1. RNA extraction and cDNA conversion*

EC and BMMSC or BMMSC-derived adipocytes mono- and co-cultures were seeded onto filters and co-cultured for 24h (as described in Section 2.9.2.). The cells were either harvested after 24h or stimulated with TNF $\alpha$  for 2h before being harvested. Cells were detached separately from either side of filters or from wells using trypsin/EDTA and then centrifuged at 400g for 5min (Section 2.4.). Total RNA was extracted using an RNeasy mini kit (Qiagen, Manchester, UK) according to the manufacturer's protocol. The RNA concentration was quantified using a Nanodrop (Thermo Scientific). 500 $\mu$ g RNA from each sample was then converted to cDNA using a Promega kit (Promega, Southampton, UK) according to the manufacturer's instructions.

Adiponectin	IFN- $\gamma$	Lipocalin-2/NGAL
Aggrecan	IGFBP-2	CCL2/MCP-1
Angiogenin	IGFBP-3	CCL7/MCP-3
Angiopoietin-1	IL-1 $\alpha$ /IL-1F1	M-CSF
Angiopoietin-2	IL-1 beta/IL-1F2	MIF
BAFF/BLyS/TNFSF13B	IL-1ra/IL-1F3	CXCL9/MIG
BDNF	IL-2	CCL3/CCL4 MIP-1 $\alpha$ / $\beta$
CD14	IL-3	CCL20/MIP-3 $\alpha$
CD30	IL-4	CCL19/MIP-3 $\beta$
CD40 Ligand/TNFSF5	IL-5	MMP-9
Chitinase 3-like	IL-6	Myeloperoxidase
Complement Component C5/C5a	IL-8	Osteopontin (OPN)
Complement Factor D	IL-10	PDGF-AA
C-Reactive Protein	IL-11	PDGF-AB/BB
Cripto-1	IL-12 p70	Pentraxin 3/TSF-14
Cystatin C	IL-13	CXCL4/PF4
Dkk-1	IL-15	RAGE
DPPIV/CD26	IL-16	CCL5/RANTES
EGF	IL-17A	RBP4
CXCL5/ENA-78	IL-18 BPa	Relaxin-2
Endoglin/CD105	IL-19	Resistin
EMMPRIN	IL-22	CXCL12/SDF-1 $\alpha$
Fas Ligand	IL-23	Serpin E1/PAI-1
FGF basic	IL-24	SHBG
KGF/FGF-7	IL-27	ST2/IL1 R4
FGF-19	IL-31	CCL17/TARC
Flt-3 Ligand	IL-32 $\alpha$ / $\beta$ / $\gamma$	TFF3
G-CSF	IL-33	TfR
GDF-15	IL-34	TGF- $\alpha$
GM-CSF	CXCL10/IP-10	Thrombospondin-1
CXCL1/GRO $\alpha$	CXCL11/I-TAC	TNF- $\alpha$
Growth Hormone	Kallikrein 3/PSA	uPAR
HGF	Leptin	VEGF
ICAM-1/CD54	LIF	Vitamin D BP

**Table 2-1: List of cytokines analysed in the human XL cytokine array kit.**

### 2.12.2. *Quantitative PCR*

A 384-well plate (Sigma) was setup to analyse genes from each culture condition. To each well 0.5µl cDNA, 0.5µl gene specific primers (Applied Biosystems, Warrington, UK), 4µl Universal PCR mastermix (Life Technologies) and 5µl DNase free water were added. Each sample was run in triplicate and three samples from separate experiments were generated for each culture condition. FAM-labelled primers for measuring IL-6, IL-6R, IL-6ST, TGFβ<sub>1-3</sub>, decorin, SOCS3, IDO1, IDO2, ICAM1, VCAM1, E-selectin, FABP4, C/EBPα, PPARγ and 18S (as a housekeeping gene) were used. All primers tested were purchased as Taqman Gene Expression Assays (Applied Biosystems; Table 2-2). The qPCR was run on a 7900HT Real Time PCR machine for 40 cycles and analysed using SDS 2.2 Software (Applied Biosystems). The threshold cycle (C<sub>T</sub>) for each gene of interest was averaged between the three sample repeats (only if the C<sub>T</sub> values were within one cycle of each other) and subtracted from the average C<sub>T</sub> value for the housekeeping gene, 18S (delta C<sub>T</sub>). The relative expression of each gene was then calculated using the formula,  $2^{-\Delta C_T}$ .

### 2.13. **Statistical analysis**

Data are expressed as mean ± SEM and the n number refers to the number of independent experiments performed per treatment. A different EC and leukocyte donor were used for each experiment. A different WJMSC and TBMSC were used for each experiment unless otherwise stated, while only 2-3 BMMSC donors were used for each series of experiments. Multi-variant data when paired were analysed using analysis of variance (ANOVA), followed by Bonferroni or Dunnett post-hoc test. Paired t-test was used when multiple parameters were grouped together even though not all conditions

were tested in every experiment, but EC mono-culture controls were performed on each occasion.  $p < 0.05$  was considered statistically significant.

<b>Primer</b>	<b>Assay ID</b>
IL-6	Hs00985639_m1
IL-6R	Hs01075666_m1
IL-6ST	Hs00174360_m1
SOCS3	Hs02330328_s1
TGF $\beta$ <sub>1</sub>	Hs00998133_m1
TGF $\beta$ <sub>2</sub>	Hs00234244_m1
TGF $\beta$ <sub>3</sub>	Hs01086000_m1
Decorin	Hs00754870_s1
ICAM1	Hs00164932_m1
VCAM1	Hs01003372_m1
E-selectin	Hs00950401_m1
PPAR $\gamma$	Hs01115513_m1
CEBP $\alpha$	Hs00269972_s1
FABP4	Hs01086177_m1
IDO1	Hs00984148_m1
IDO2	Hs01589373_m1
IL-10	Hs00961622_m1

**Table 2-2: List of primers used for qPCR.**

### **3. Isolation and characterisation of MSC from different tissue sources**

### 3.1. Introduction

MSC can be isolated from most human tissues and while BMMSC remain the most widely studied and accessible source, recently MSC isolated from umbilical cords and trabecular bone have gained considerable interest. This is because these tissues generate a greater yield of highly proliferative MSC that have similar immunosuppressive properties as BMMSC (Mafi *et al.*, 2011; Baksh *et al.*, 2007). Despite intense research, a specific MSC phenotype has not yet been defined. Therefore, the ISCT have recommended minimal criteria for classifying MSC based on their expression of an array of cell surface markers and their capacity to differentiate into adipocytes, osteoblasts and chondrocytes (Dominici *et al.*, 2006). While these criteria can be applied to MSC from all tissue sources, it is often the case that between MSC populations there are differences in MSC marker expression and in their differentiation capacity (Russell *et al.*, 2010). Recently, CD146 and CD271 have been identified as markers exclusively expressed by MSC with greater clonogenic potential and immunomodulatory capacity (Maijenburg *et al.*, 2012; Kuci *et al.*, 2010). However, there are currently no functional markers that can be used to enrich for MSC. Interestingly, while MSC from different sources show phenotypic differences, they all exert similar functional effects such as the ability to suppress T-cell proliferation (Russell *et al.*, 2010; Christodoulou *et al.*, 2013). This suggests that these phenotypic differences have no functional effects on the cells.

MSC are typically isolated from solid tissue by either culturing tissue explants or enzymatic digestion of tissue fragments. In this chapter we optimised the isolation and culture of MSC from human umbilical cord WJ, a readily available source of MSC. We

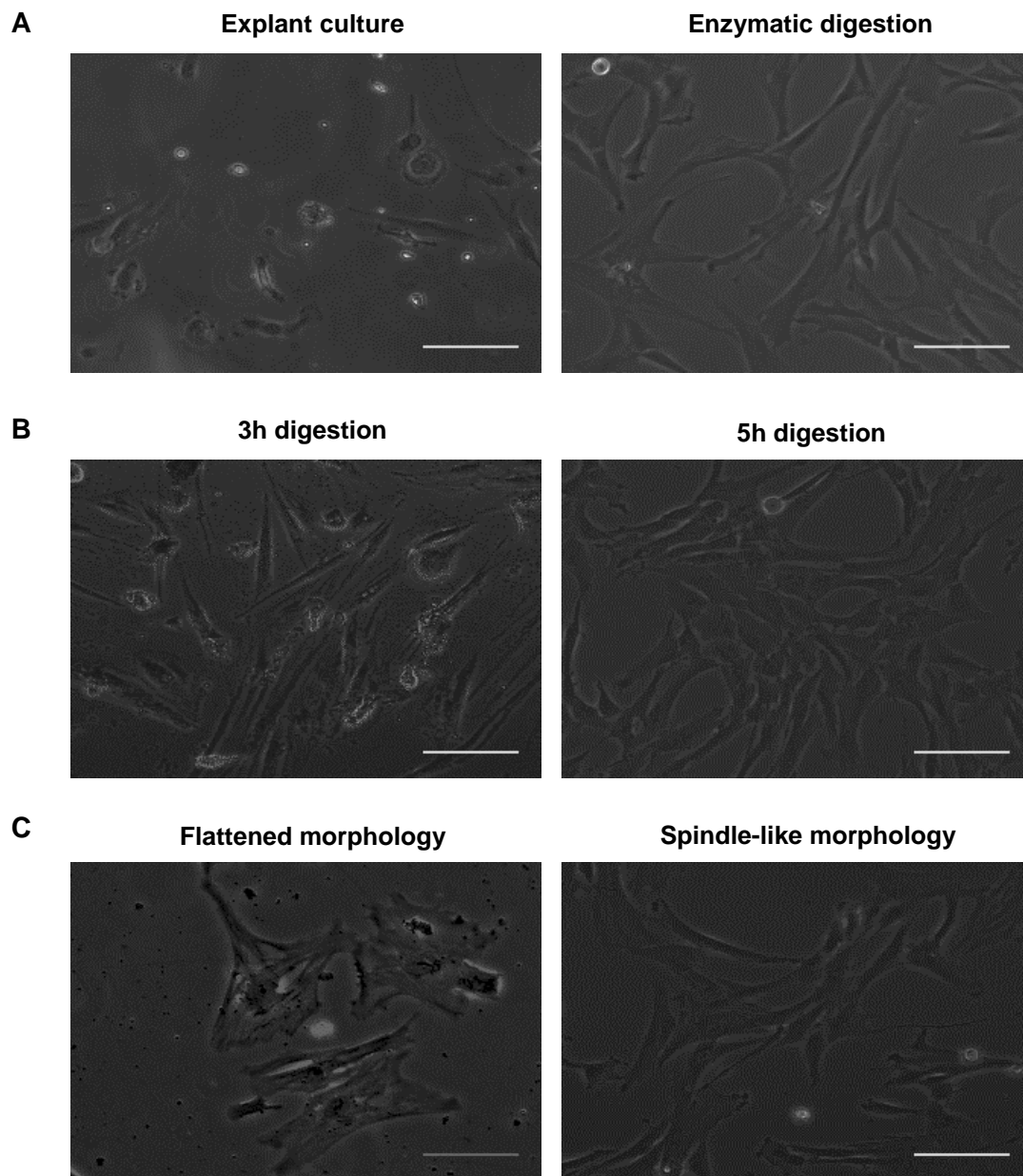
then compared the morphology, surface marker profile and adipogenic and osteoblastic differentiation potential of WJMSC isolated by the optimal method, to MSC isolated in house from TB by explant culture and to commercially-available, pre-defined BMMSC. This was the first step in examining whether phenotypic differences existed between MSC populations, which might be linked to functional differences in their immunomodulatory capacity.

## **3.2. Results**

### *3.2.1. Comparison of different methods for isolating WJMSC*

The morphology of MSC isolated from solid tissues can serve as a good indicator of the viability of the cells after isolation as MSC exhibit altered morphology when exposed to stressful culture conditions (McCormick *et al.*, 2006). Therefore, we examined the morphology of WJMSC isolated by explant culture or enzymatic digestion after 14 days in culture. Cells isolated by explant culture were very heterogeneous and the majority of cells were small and irregularly shaped (Figure 3-1A). Only a few cells within the culture exhibited a spindle-like, fibroblastic morphology, characteristic of MSC (Figure 3-1A). In contrast, WJMSC isolated by enzymatic digestion contained a greater proportion of cells with the typical fibroblastic morphology suggesting that MSC isolated by this method were more viable than those isolated by explant culture (Figure 3-1A). As the enzymatic digestion method yielded a greater proportion of cells with MSC-like morphology than the explant culture method, this method was used for all subsequent work.





**Figure 3-1: Comparison of the morphology of WJMSC isolated by explant culture and enzymatic digestion.** MSC (passage 0) were isolated from umbilical cord Wharton's jelly by cutting the tissue into small fragments and placing them into culture flasks to allow MSC to migrate from the tissue and adhere to the plastic (Explant culture). Alternatively, tissue fragments were incubated with an enzymatic cocktail for 5h and the cell suspension obtained was then seeded onto culture flasks (Enzymatic digestion). (A) Representative phase contrast images of WJMSC isolated using the explant culture and enzymatic digestion methods respectively, 14 days post-isolation. (B) Representative phase contrast images of WJMSC morphology 14 days after enzymatic digestion for 3h or 5h. (C) Representative images of the two distinct morphologies; flattened vs. spindle shaped observed 14 days after enzymatic digestion for 5h. These images are representative of n= 2 experiments. All images were taken at p0 at 20X magnification. Scale bars represent 10 $\mu$ m.

Next, we tested the optimum time period to digest the umbilical cord tissue to yield the maximum number of WJMSC. Umbilical cord tissue digestion was performed for 3h or 5h and the morphology was examined 14 days post-isolation. WJMSC obtained after 5h tissue digestion yielded a greater proportion of cells with characteristic fibroblastic morphology than after 3h digestion (Figure 3-1B). WJMSC isolated after 3h digestion were more flattened and thin than typical MSC (Figure 3-1B). In addition, the cell suspension obtained after 3h digestion was significantly more viscous than after 5h and was harder to filter (to remove larger tissue fragments). Thus, 5h enzyme incubation was chosen as the optimum length of time to digest the tissue.

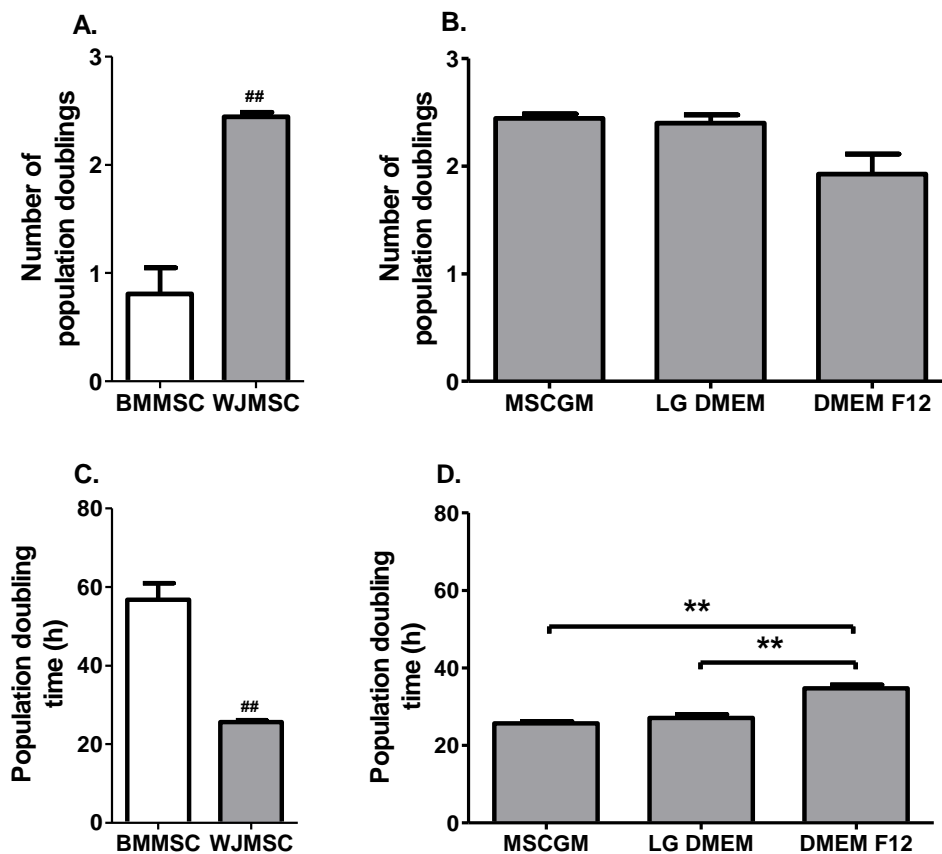
WJMSC cultures obtained by enzymatic digestion contained cell types with two distinct morphologies. Cells with a flattened, egg-shaped morphology and typical spindle-like morphology were observed (Figure 3-1C). Initially, there were a greater number of MSC with the flattened morphology during early culture and fewer spindle shaped MSC. The spindle-like MSC subsequently dominated the culture and continued to propagate with passage and the cells with flattened morphology were not observed after the first passage.

### *3.2.2. Proliferation kinetics of BMMSC and WJMSC*

Subsequently, we assessed the proliferation of WJMSC cultured in different types of cell culture media and compared to BMMSC cultured in MSCGM in order to identify the optimum media for WJMSC expansion. The proliferation kinetics (number of population doublings, PD and the population doubling time, PDT) were determined after culturing WJMSC in MSCGM, LG DMEM and DMEM F12 as these media have

previously been used to culture WJMSC (Conconi, 2011). There was no significant difference in the number of PD undergone by p3 WJMSC cultured in the different media (Figure 3-2B). However, WJMSC in all culture media underwent a significantly greater number of PD than p5 BMMSC cultured in MSCGM (Figure 3-2A). p3 WJMSC cultured in MSCGM and LG DMEM underwent approximately 2.5 PD and 1.8PD in DMEM F12 compared to 1.0 PD by BMMSC after 5 days in culture (Figure 3-2A and B).

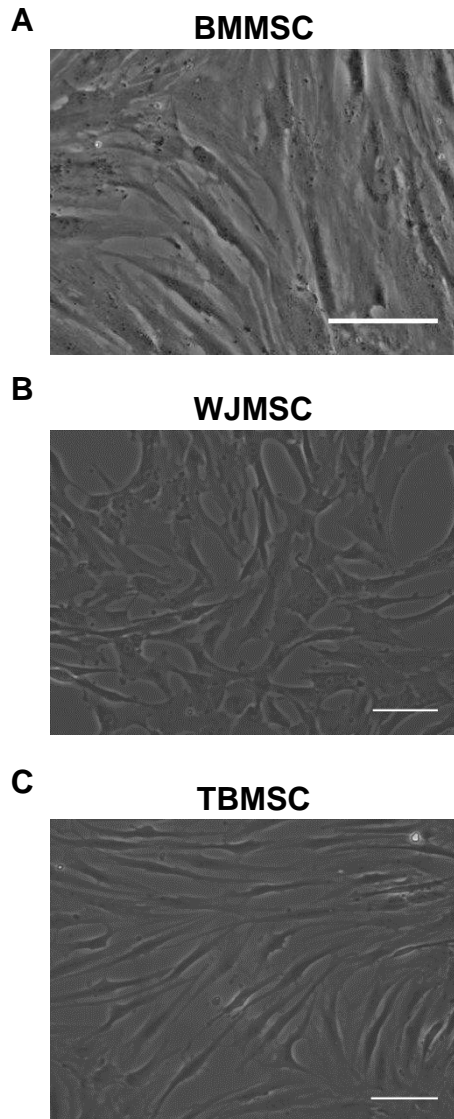
Mean PDT was found to be significantly higher in BMMSC (Figure 3-2C) than WJMSC cultured in all media (Figure 3-2D). BMMSC doubled after ~60h at p5, while WJMSC cultured in MSCGM and LG DMEM doubled in under 25h (Figure 3-2C). PDT of WJMSC cultured in DMEM F12 was significantly higher (~33h) than MSC cultured in MSCGM or LG DMEM (Figure 3-2D). Interestingly, WJMSC had faster proliferation kinetics than BMMSC in all media tested. However, after comparing the different media, LG DMEM was selected as the culture media for WJMSC expansion as the proliferation kinetics were faster than MSC cultured in DMEM F12. Furthermore, LG DMEM had the same effect on WJMSC proliferation as MSCGM but the media was significantly cheaper. The proliferation kinetics of TBMSC were not examined as they proliferated too slowly for such analysis to be performed, taking up to 30 days to reach confluence. TBMSC were cultured in LG DMEM for comparison to WJMSC.



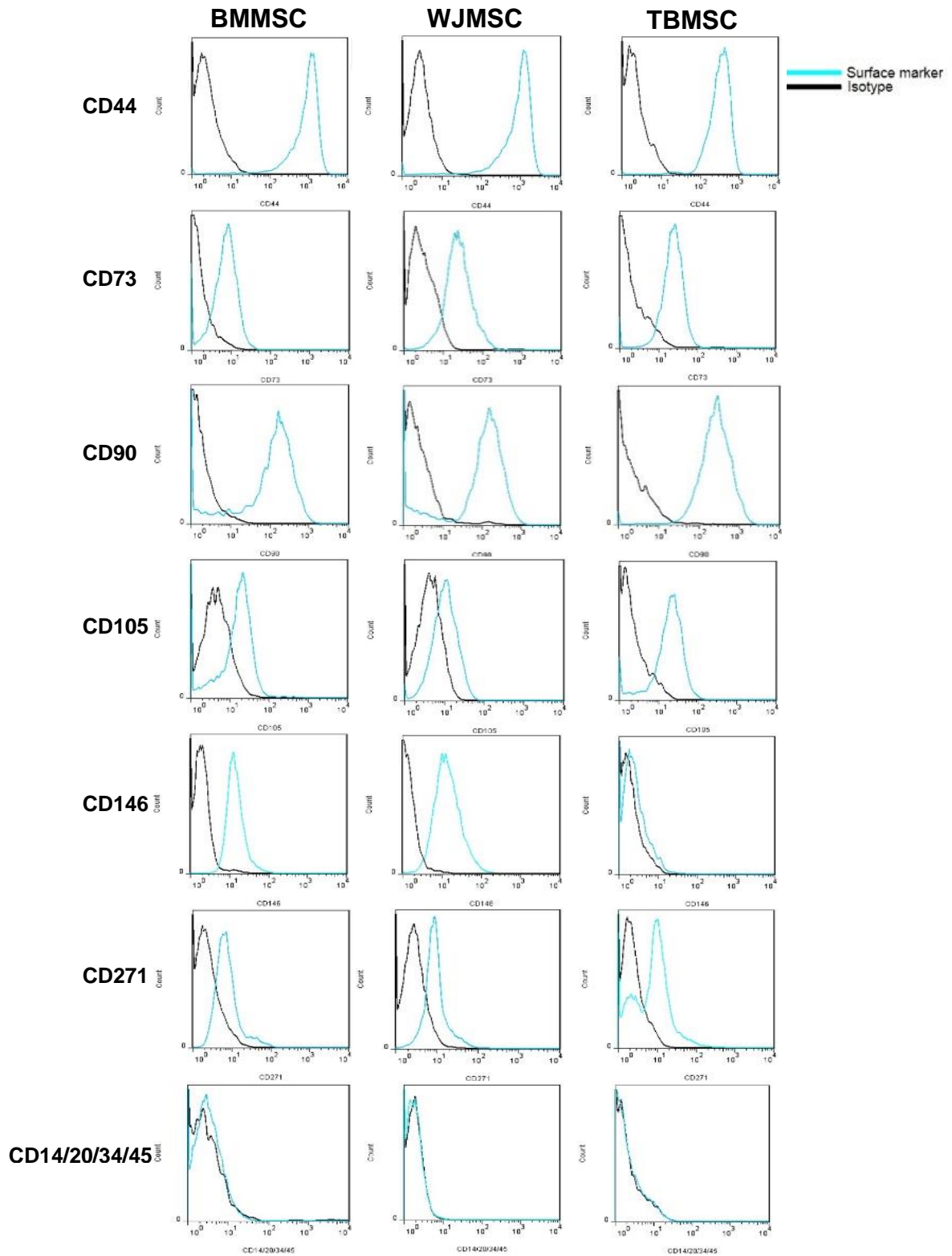
**Figure 3-2: Proliferation kinetics of BMMSC and WJMSC in different culture media.** (A,B) The number of population doublings and (C,D) the population doubling time of (A,C) p5 BMMSC and p3 WJMSC cultured in MSCGM and (B,D) p3 WJMSC cultured in MSCGM, LG DMEM and DMEM F12 were analysed after 5 days in culture. Data are mean  $\pm$  SEM from  $n=3$  experiments. In A and C, unpaired t-test showed a significant difference in the number of PD and PDT between BMMSC and WJMSC; <sup>##</sup> =  $p<0.01$ . In B, ANOVA showed a significant effect of culture media on the number of PD undergone by WJMSC. In D, ANOVA showed a significant effect of culture media on the PDT of WJMSC,  $p<0.01$ . <sup>\*\*</sup> =  $p<0.01$  by Bonferroni post-test. A different BMMSC and WJMSC donor was used per experiment.

### *3.2.3. Comparison of the phenotype of different MSC populations:*

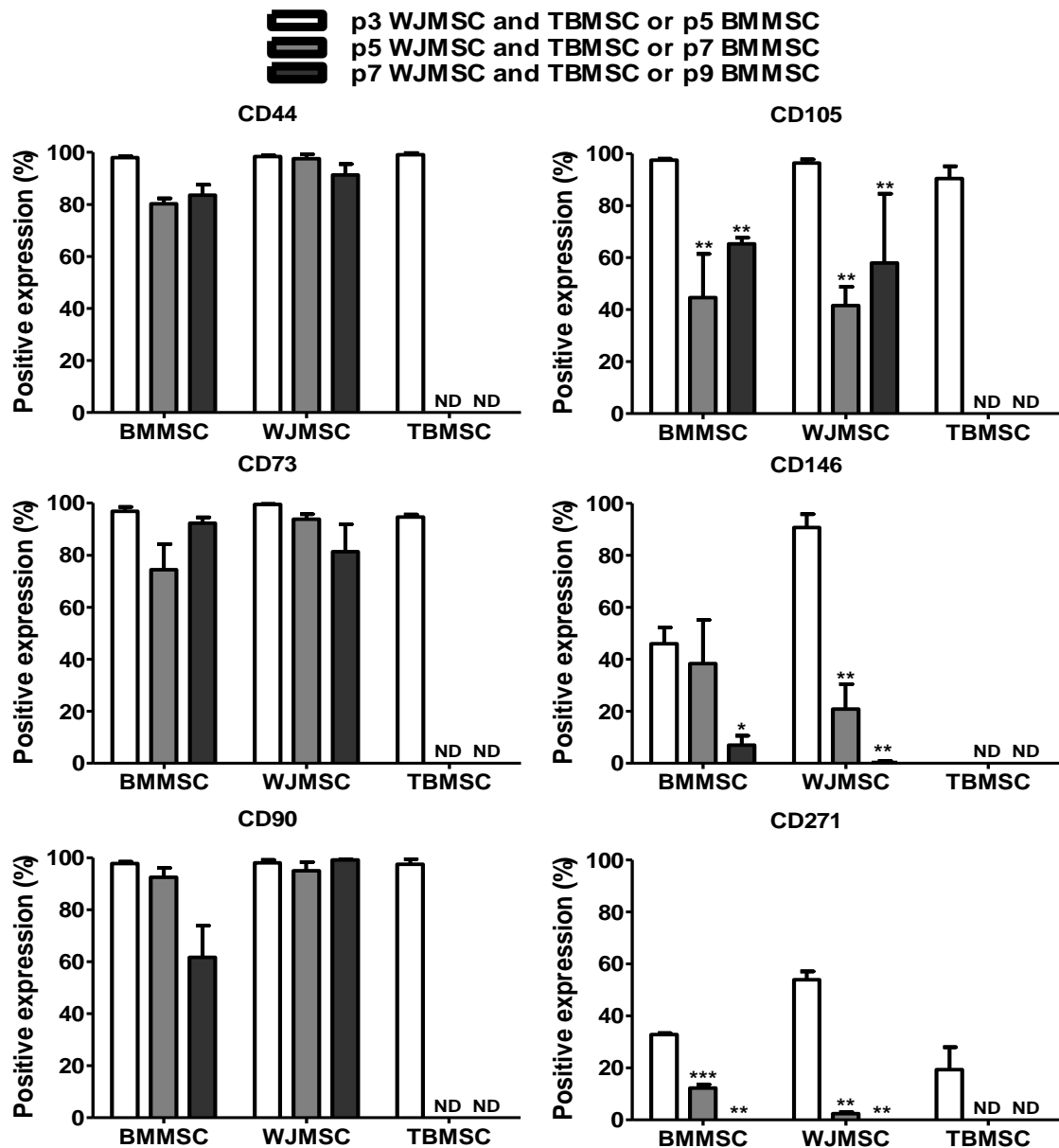
We next assessed whether the isolated MSC fulfil the ISCT criteria. WJMSC (isolated by digestion) and TBMSC (isolated by explant) at p3 exhibited the same fibroblastic morphology as p5 BMMSC (Figure 3-3). The expression of the MSC marker panel on p3 WJMSC and TBMSC was then tested and compared to p5 BMMSC. The expression of MSC markers CD44, CD73, CD90, CD105, CD146, and CD271 was analysed by flow cytometry. Expression of negative markers CD14, CD20, CD34 and CD45 were also examined. All 3 MSC types, showed high expression of CD44, CD73, CD90 and CD105 (Figure 3-4). Interestingly, only BMMSC and WJMSC expressed CD146 while all 3 MSC populations expressed CD271 (Figure 3-4). Minimal contamination by endothelial and haematopoietic progenitors (based on CD14, CD20, CD34 and CD45) was observed in all cultures (Figure 3-4). The expression of the marker panel was measured on p5, p7 and p9 BMMSC and p3, p5 and p7 WJMSC to examine whether prolonged culture altered their expression profile. Expression on TBMSC was only assessed at p3 as they could not be expanded further. It was observed that 90-100% BMMSC and WJMSC retained CD44, CD73 and CD90 expression over passage (Figure 3-5). A significant reduction (40-50%) in the number of CD105 expressing cells was observed with passage. ~90% WJMSC expressed CD146 at p3 but this diminished significantly with passage and was almost completely lost by p5 (Figure 3-5). Similarly, the expression of CD271 was higher on p3 WJMSC (55%) than BMMSC (30%) and TBMSC (25%) and significantly reduced with passage (Figure 3-5). Collectively, these data indicate that WJMSC and TBMSC fulfil the criteria for MSC marker expression and that WJMSC show greater level of CD146 and CD271 expression.



**Figure 3-3: The morphology of BMMSC, WJMSC and TBMSC.** Representative phase contrast images of (A) p5 BMMSC, (B) p3 WJMSC and (C) p3 TBMSC. Images are representative of N= 5-7 MSC donors. Images were taken at (A) 20X or (B,C) 10X magnification. Scale bar represents 10 $\mu$ m.



**Figure 3-4: Expression of MSC markers by BMMSC, WJMSC and TBMSC.** Representative histogram plots of CD44, CD73, CD90, CD105, CD146, CD271 and negative markers (CD14/CD20/CD34/CD45 combined) expression on p5 BMMSC, p3 WJMSC and p3 TBMSC compared to isotype control treated MSC. Images are representative of n= 3 experiments. A different BMMSC, WJMSC and TBMSC donor was used per experiment.



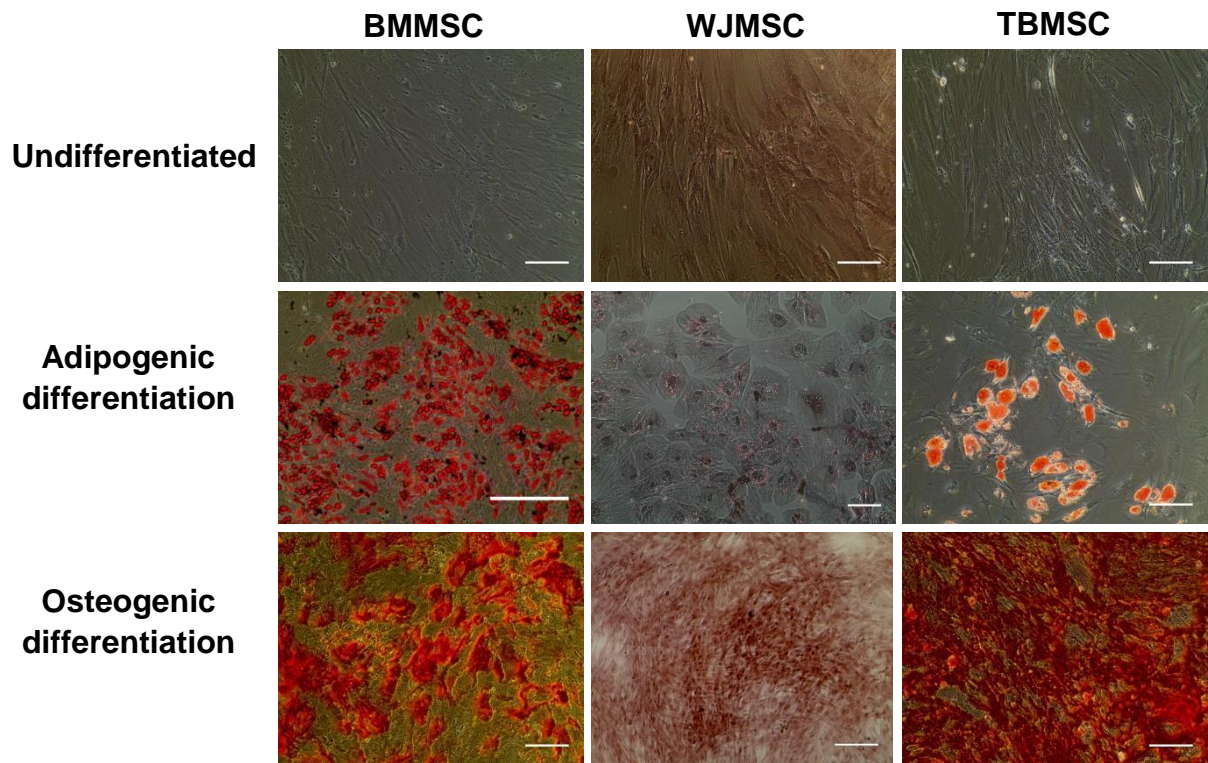
**Figure 3-5: Expression of MSC markers by BMMSC, WJMSC and TBMSC at different passages.** The percentage of cells expressing CD44, CD73, CD90, CD105, CD146 and CD271 by p5 BMMSC, p3 WJMSC and p3 TBMSC was analysed compared to isotype control treated MSC. Data are mean  $\pm$  SEM from  $n=3$  independent experiments. ANOVA showed a significant effect of passage on expression of CD105, CD271 and CD146,  $p<0.05$ . \* =  $p<0.05$  and \*\* =  $p<0.01$  compared to p5 BMMSC or p3 WJMSC by Bonferroni post-test. ND = not determined. 3 different WJMSC and TBMSC donors and 2 different BMMSC donors were used in this set of experiments.



#### *3.2.4. Comparison of the differentiation potential of the different MSC populations*

The capacity of all MSC populations to differentiate into adipocytes and osteoblasts was also examined, as this was stipulated by the ISCT to be a functional requirement for an MSC. p5 BMMSC, p3 WJMSC and p3 TBMSC exhibited the capacity to differentiate into adipocytes, confirmed by Oil Red O staining of their lipid droplets (Figure 3-6). However, WJMSC contained fewer lipid droplets than BMMSC and TBMSC (Figure 3-6). BMMSC-derived adipocytes contained larger lipid vacuoles than TBMSC or WJMSC (Figure 3-6). Differentiation in adipocyte induction media was carried out for 21 days as lipid droplets in the WJMSC could only be detected at this point. In contrast, lipid droplets were detectable in BMMSC and TBMSC cultures after 7-10 days in culture, indicating that these MSC populations had a greater propensity to differentiate down the adipogenic lineage.

Osteogenic differentiation was carried out for 14 days and assessed by Alizarin Red staining of mineralised calcium deposits produced by the cells. All MSC types generated large calcium deposits after 14 days differentiation indicating that they could all differentiate down the osteogenic lineage (Figure 3-6). TBMSC contained more calcium deposits that were larger in size than BMMSC or WJMSC (Figure 3-6). WJMSC and BMMSC had capacity to differentiate down the osteogenic lineage. Chondogenic differentiation potential of MSC from the different sources was not examined in this thesis.



**Figure 3-6: Differentiation of BMMSC, WJMSC and TBMSC towards adipocytes or osteoblasts.** Representative phase-contrast images of p5 BMMSC, p3 WJMSC and p3 TBMSC and MSC-derived adipocytes and osteoblasts are shown. Lipid droplets in the adipocytes were stained with Oil Red O. Calcium deposits shown in the osteoblast culture were stained with Alizarin Red. Images are representative of n= 3 independent experiments. Images were taken at 20X magnification (except for adipogenically differentiated and WJMSC which were taken at 4X). Scale bar represents 10 $\mu$ m. A different BMMSC, WJMSC and TBMSC donor was used per experiment.

### 3.3. Discussion

In this chapter, we developed new, optimal isolation and culture conditions for WJMSC. We subsequently characterised BMMSC, WJMSC and TBMSC based on expression of MSC markers and differentiation potential. Commercially available BMMSC at p5, p3 WJMSC isolated by enzymatic digestion and p3 TBMSC isolated by explant culture expressed the full panel of MSC markers and lacked expression of negative markers. BMMSC and WJMSC retained expression of these markers over further passage suggesting that they didn't spontaneously differentiate during culture. Analysis of marker expression by later passage TBMSC could not be performed as the cells could not be expanded beyond p3. BMMSC, WJMSC and TBMSC exhibited osteoblastic and adipogenic differentiation potential albeit to varying degrees based on staining and appearance of the differentiated cells. Therefore, MSC isolated from WJ and TB met the ISCT criteria with the exception of chondrogenic differentiation potential of the MSC which was not assessed.

One issue with defining MSC by this criteria is that it is very generic. In fact, other stromal cell populations such as human dermal fibroblasts also express CD44, CD73, CD90 and CD105 (Alt *et al.*, 2011; Halfon *et al.*, 2011) and are capable of differentiating into adipocytes and osteoblasts when cultured in appropriate media (Haniffa *et al.*, 2007). This indicates that this criterion is not specific to MSC alone and will need to be used in conjunction with functional studies to determine whether isolated cells are in fact MSC. There are currently no MSC-specific cell surface markers or functional markers that can be used to distinguish MSC from other tissue-resident stromal populations that potentially contaminate MSC cultures.

WJMSC isolated by enzymatic digestion initially exhibited two distinct morphologies; flattened and spindle-like. It has previously been reported that three subpopulations of BMMSC arise in culture soon after isolation; small, spherical rapidly dividing cells, spindle-shaped fibroblastic cells and flattened cuboidal cells that had a low proliferation capacity (Sekiya *et al.*, 2002; Battula *et al.*, 2009; Mets and Verdonk, 1981). The flattened BMMSC were identified as mature MSC that showed early signs of commitment to the osteoblastic lineage and as such had a reduced proliferation and differentiation potential compared to fibroblastic MSC (Colter *et al.*, 2000, 2001). Here, WJMSC showed similar heterogeneity in culture at p0, but the flattened WJMSC did not survive in culture as their proliferation rate appeared to be much slower than the fibroblastic MSC. Consequently, the flattened MSC population were not observed after p0 and a uniform population of fibroblastic MSC was obtained by p3.

MSC from different tissue sources have been shown to be phenotypically and functionally similar, although they may exhibit differences in their capacity to proliferate and differentiate. For instance, umbilical cord-derived MSC had a greater proliferative and clonogenic capacity than BMMSC and ADMSC despite exhibiting similar surface marker expression and differentiation capabilities (Jin *et al.*, 2013; Christodoulou *et al.*, 2013; Baksh *et al.*, 2007). We also observed that WJMSC proliferated significantly faster than BMMSC based on the number of PD and PDT of both MSC populations.

Although trilineage differentiation is a key phenotypic feature of MSC, we observed that BMMSC, WJMSC and TBMSC exhibited differences in their capacity to

differentiate into adipocytes and osteoblasts. BMMSC and TBMSC differentiated into adipocytes with larger lipid droplets than WJMSC. In contrast, TBMSC showed a greater degree of calcium deposition than BMMSC and WJMSC after differentiation into osteoblasts, as confirmed by staining. This suggests that there are phenotypic differences between MSC from different tissue sources.

Passaging MSC may also affect their phenotype as changes in cell morphology, size, surface marker expression (CD44, CD105, CD146 and CD271) and differentiation potential have previously been reported [reviewed by (Eggenhofer *et al.*, 2014)]. Indeed, we observed that expression of CD105, CD146 and CD271 significantly diminished with passage, particularly for WJMSC. As CD271 and CD146 are markers of MSC that exhibit greater colony forming unit capacity and suppression of mitogen induced T-cell proliferation (Maijenburg *et al.*, 2012; Kuci *et al.*, 2010), the decrease in expression over passage suggest that BMMSC and WJMSC may lose their growth potential and immunomodulatory capacity with passage. In addition, previous studies showed that BMMSC and ADMSC underwent growth arrest at earlier passage (p11-12) than umbilical cord-derived MSC (beyond p18) and had a significantly lower growth rate (Jin *et al.*, 2013; Christodoulou *et al.*, 2013). Additionally, BMMSC had a decreased adipogenic differentiation potential and an increase in osteogenic differentiation capacity with passage (p5-10) (Wagner *et al.*, 2008). In contrast, WJMSC retained their ability to differentiate down the characteristic mesodermal lineages (adipocyte, osteoblast and chondrocyte) as far as p15 (Chen *et al.*, 2014). These studies suggest that the MSC phenotype is altered in culture, with WJMSC retaining their phenotypic characteristics for longer than BMMSC. Based on our findings and those of other groups, WJMSC may be a better source than BMMSC for

therapy as they grow faster and express higher levels of CD146 and CD271. However, given that we observed that WJMSC displayed a poor differentiation capacity compared to the BMMSC and TBMSC, this will greatly limit their therapeutic potential. It also raises the question whether WJMSC are in fact “true” MSC if they exhibit reduced capacity to differentiate down the adipogenic and osteogenic lineages.

In conclusion, we observed that MSC from different sources exhibit differences in their growth potential, surface marker expression and differentiation capacity. Indeed, WJMSC had a greater growth potential, retention of MSC marker expression over passage and osteoblastic differentiation potential than BMMSC and TBMSC. Whether these phenotypic differences in MSC populations observed translated into functional differences in their capacity to regulate neutrophil recruitment in response to inflammation was assessed in the following chapter.

## **4. Capacity of MSC from different sources to regulate neutrophil recruitment to vascular EC**

#### 4.1. Introduction

Numerous *in vitro* and *in vivo* studies have demonstrated that MSC from different anatomical sites are capable of directly modifying leukocyte proliferation and effector functions [reviewed by (Le Blanc and Mougiakakos, 2012; Munir and McGettrick, 2015)]. However, these studies have not considered the possibility that MSC may also directly influence the recruitment of the inflammatory infiltrate. Previously we have shown that cross-talk between BMMSC and EC reduced the endothelial response to cytokines, suppressing leukocyte adhesion and migration (Luu *et al.*, 2013). This evidence suggests that MSC may be endogenous regulators of recruitment, limiting entry of leukocytes into inflamed tissues. Whether MSC from different sources have the same capacity to regulate recruitment or whether this is a tissue-specific feature of BMMSC was the main focus of this chapter.

Recent evidence suggests that the MSC niche varies at different anatomical sites, likely due to differences in the levels of locally released factors (e.g. cytokines) and interactions with neighbouring stromal cells. Differences in the tissue microenvironment may lead to functional differences between MSC populations (Magatti *et al.*, 2008; Wolbank *et al.*, 2007; Chen *et al.*, 2011; Chang *et al.*, 2006). However, isolation and *ex vivo* expansion of MSC has been shown to adversely affect their immunomodulatory responses, therefore any tissue-specific differences may be lost during culture expansion (Li *et al.*, 2012; Giuliani *et al.*, 2011; Yu *et al.*, 2014; Bortolotti *et al.*, 2015). Indeed, several studies have reported that MSC from different tissues that were expanded in culture exerted similar suppressive effects on leukocyte



responses, e.g. T-cell proliferation (Roemeling-van Rhijn *et al.*, 2013; Puissant *et al.*, 2005; Chang *et al.*, 2006; Trivanović *et al.*, 2013).

MSC from different tissues sources may also induce their immunosuppressive effects to different extents (Prasanna *et al.*, 2010). For instance, WJMSC express different levels of immunosuppressive factors (hepatocyte growth factor, IDO and PGE<sub>2</sub>) than BMMSC (Prasanna *et al.*, 2010; Deuse *et al.*, 2011) which may lead to differences in their capacity to regulate immune cell responses. While most studies implicate soluble mediators as the main drivers for immunosuppression, recently it has been suggested that direct cell-cell contact may be required for inducing or enhancing MSC effector functions directly on leukocyte functions (Brandau *et al.*, 2014; Spaggiari *et al.*, 2008; Sotiropoulou *et al.*, 2006). While the effects of MSC on leukocyte effector functions have been extensively explored, there is little experimental evidence on the mechanism by which MSC crosstalk with EC regulates leukocyte recruitment. Whether MSC from different tissues utilise distinct mechanisms (direct contact vs. soluble mediators) to regulate neutrophil recruitment was assessed in this chapter. We compared the capacity of MSC from different tissues namely BMMSC, WJMSC and TBMSC to regulate the recruitment of neutrophils to cytokine-stimulated EC. Two distinct *in vitro* models (a direct co-culture model with MSC incorporated in the EC monolayer and a Transwell filter model with the different cell types cultured on opposite sides of the porous filter) were used to study the effects of MSC from different tissue sources on the capacity of EC to support neutrophil recruitment during inflammation.

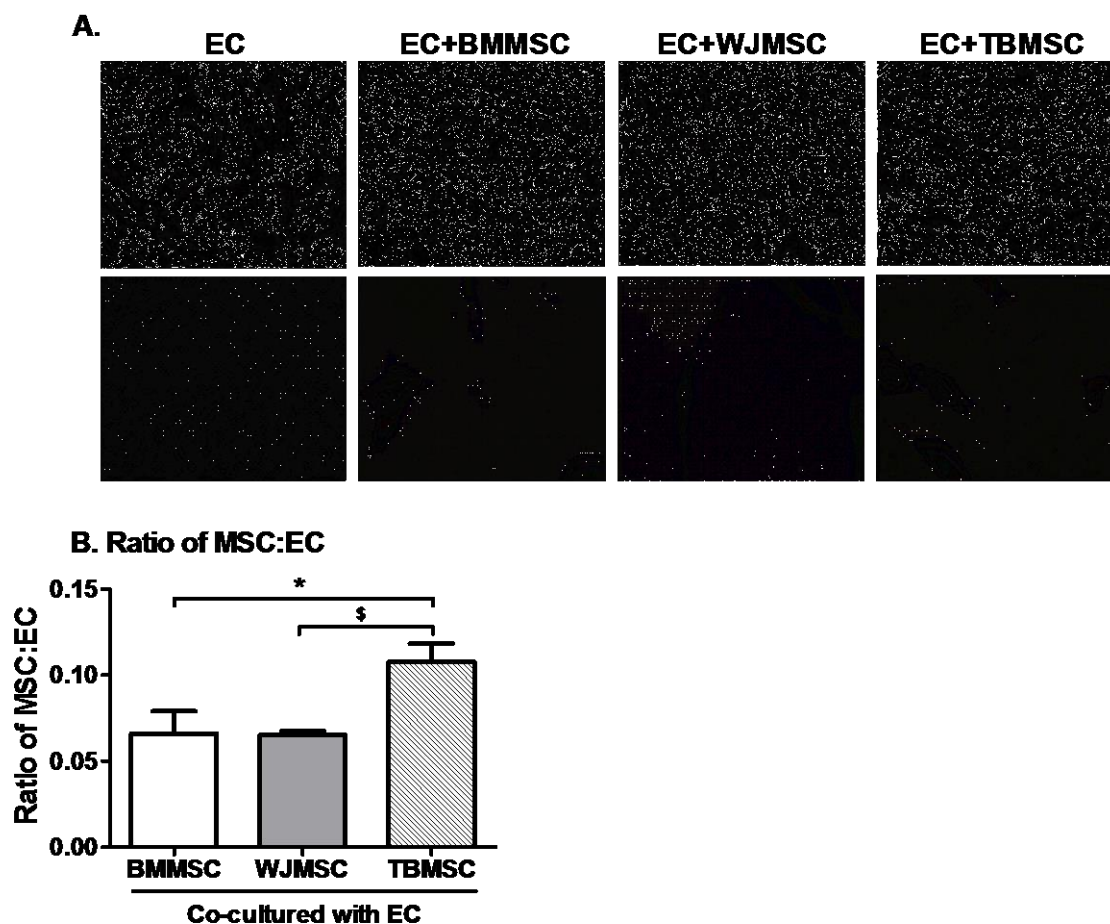
## 4.2. Results

### 4.2.1. Ability of MSC from different sources to incorporate into endothelium

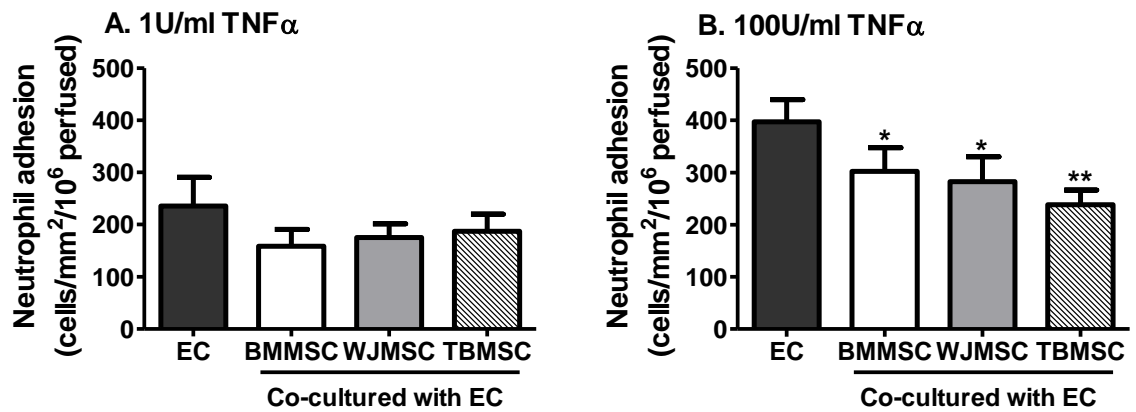
We previously showed that direct co-culture of BMMSC and EC at a ratio of 1:25 in a microchannel slide flow-based adhesion assay suppressed neutrophil adhesion and migration (Luu *et al.*, 2013). Utilising the same model we tested the ability of BMMSC, WJMSC and TBMSC to adhere to EC by adding the same density of MSC and observing how many bound and incorporated into the EC monolayer. All MSC populations adhered to the EC and lost their characteristic fibroblastic morphology (Figure 4-1A). Importantly, the integrity of the EC monolayer was not compromised by the addition of MSC as there were no gaps around the area where the MSC had attached (Figure 4-1A). Interestingly, significantly more TBMSC adhered to the EC than WJMSC and BMMSC respectively (Figure 4-1B).

### 4.2.2. Effects of MSC from different sources cultured in direct contact with EC on neutrophil recruitment

Subsequently, we tested the effect of different MSC on neutrophil recruitment to EC using the microchannel slide model (as described in Section 2.9.) in response to low and high grade inflammation. The cells were stimulated with either 1U/ml (low grade inflammation) or 100U/ml (high grade inflammation) TNF $\alpha$  for 4h. At 100U/ml TNF $\alpha$ , neutrophil adhesion was higher compared to 1U/ml TNF $\alpha$  in EC mono-cultures (Figure 4-2). Co-culture with all MSC populations reduced neutrophil adhesion compared to EC mono-cultures at both concentrations of TNF $\alpha$ , but the suppression was only statistically significant after stimulation with 100U/ml TNF $\alpha$  (Figure 4-2).



**Figure 4-1: Adhesion of different MSC types to EC in microchannel slides.** Fluorescently labelled MSC (BMMSC, WJMSC and TBMSC) were added to a confluent monolayer of HUVEC in a microchannel slide and left to adhere for 1h. Non-adherent MSC were washed off and remaining cells were cultured for 24h. Images were taken after a flow based adhesion assay was performed. (A) Representative phase-contrast images and corresponding fluorescent images of EC mono-cultures and MSC co-cultures. Images were taken at 20X magnification. (B) The ratio of adherent MSC:EC for the 3 MSC populations. Data are mean  $\pm$  SEM from  $n=3$  experiments. ANOVA showed a significant effect of MSC source on MSC:EC ratio,  $p<0.05$ . \* =  $p<0.05$  and \$ =  $p=0.053$  by Bonferroni post-test. A different EC and BMMSC, WJMSC and TBMSC donor was used in each experiment.

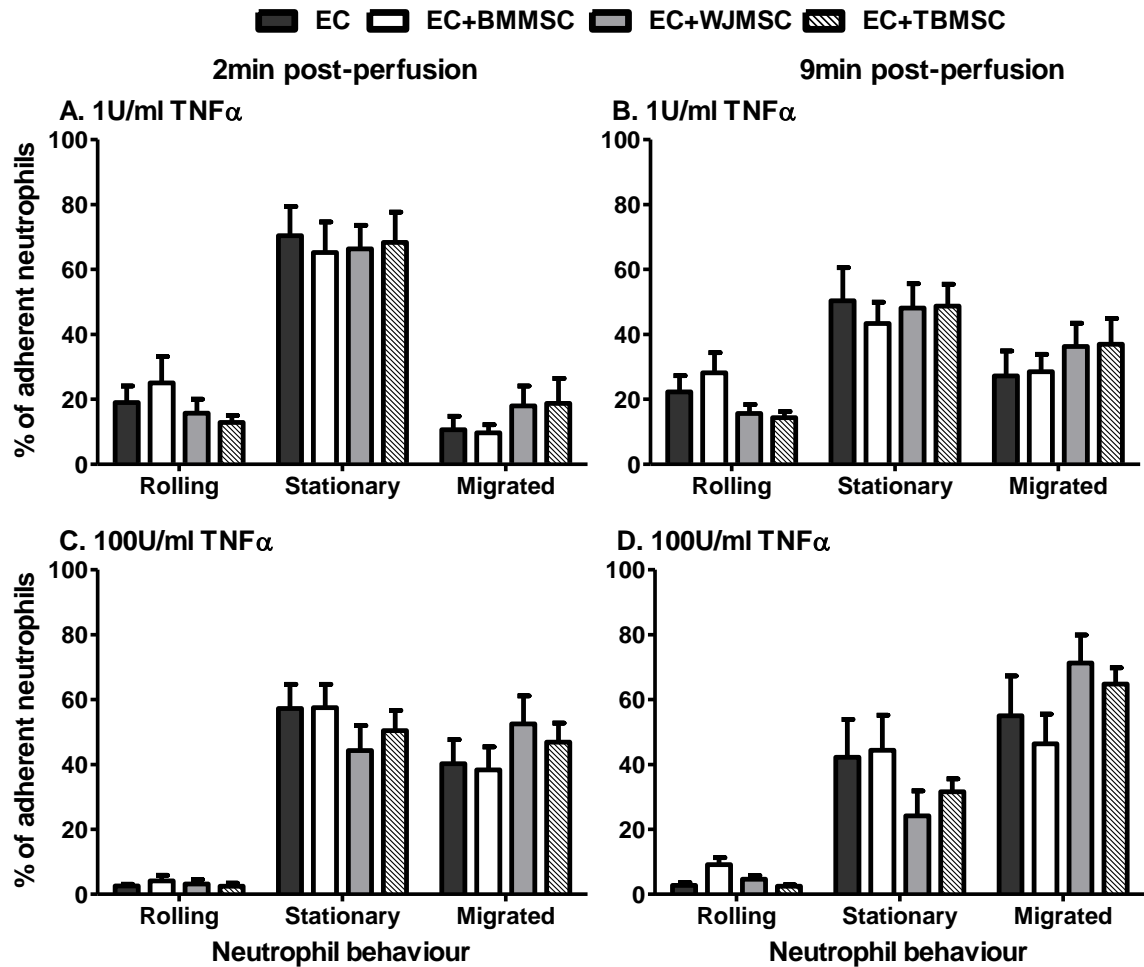


**Figure 4-2: Effect of MSC co-culture on neutrophil adhesion to inflamed EC in microchannel slides.** EC mono-cultures and MSC co-cultures were stimulated with either (A) 1U/ml or (B) 100U/ml TNF $\alpha$  for 4h and neutrophil adhesion was analysed. Data are mean  $\pm$  SEM from n= 5 experiments. In B, ANOVA showed a significant effect of culture conditions on neutrophil adhesion,  $p < 0.01$ . \* =  $p < 0.05$  and \*\* =  $p < 0.01$  compared to EC mono-cultures by Dunnett post-test. A different EC and neutrophil donor was used per experiment. 3 different BMMSC and 5 different WJMSC and TBMSC donors were used in this set of experiments.

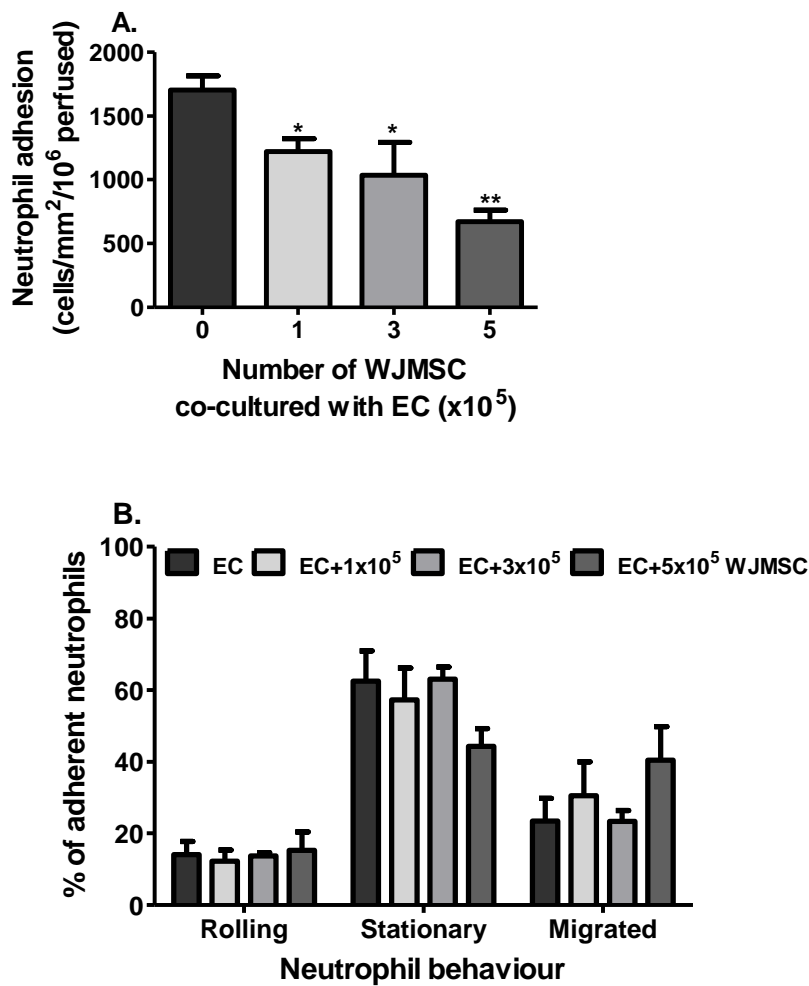
In response to low dose TNF $\alpha$ , a higher percentage of adherent neutrophils were rolling and stationary and less migrated compared to high dose TNF $\alpha$  at 2min and 9min post-perfusion, however, it was not statistically significant (Figure 4-3). At both concentrations of TNF $\alpha$ , neutrophil migration tended to be higher at 9min post-perfusion than 2min in EC mono-cultures (Figure 4-3). However, at both time points co-culture with MSC did not significantly alter neutrophil behaviour compared to the EC mono-cultures (Figure 4-3). As the suppressive effect of MSC on neutrophil adhesion was greater at 100U/ml TNF $\alpha$  this concentration was selected for all subsequent experiments. Furthermore, as migration was higher at 9min post-perfusion, all subsequent behavioural analysis was performed at this time point.

#### *4.2.3. Effects of culturing MSC from different sources on opposite side of filters to EC on neutrophil recruitment*

Next, we cultured MSC (from all 3 sources) and EC on opposite sides of a porous filter and assessed neutrophil recruitment using the flow based adhesion assay (described in Section 2.9.2.). To find the optimum ratio of MSC to EC to obtain maximum suppression of neutrophil recruitment,  $1 \times 10^5$ ,  $3 \times 10^5$  or  $5 \times 10^5$  WJMSC were seeded onto filters and co-cultured with EC for 24h. Co-culture with WJMSC was found to significantly reduce neutrophil adhesion in a dose-dependent manner relative to the EC control (Figure 4-4A). However, once again co-culture had no significant effect on neutrophil behaviour compared to the EC control (Figure 4-4B). As the greatest suppressive effect was obtained with  $5 \times 10^5$  MSC, this density was used for the remainder of the study.



**Figure 4-3: Effect of MSC co-culture on the behaviour of adherent neutrophils in microchannel slides.** EC mono-cultures and MSC co-cultures were stimulated with either (A,B) 1U/ml or (C,D) 100U/ml TNF $\alpha$  for 4h. The behaviour of adherent neutrophils was analysed at (A,C) 2min and (B,D) 9min post-perfusion. Neutrophil behaviour was expressed as the percentage of adherent neutrophils that were rolling, stationary and migrated. Data are mean  $\pm$  SEM from n= 5 experiments. ANOVA showed no significant effect of MSC co-culture on neutrophil behaviour. A different EC and neutrophil donor was used per experiment. 3 different BMMSC and 5 different WJMSC and TBMSC donors were used in this set of experiments.



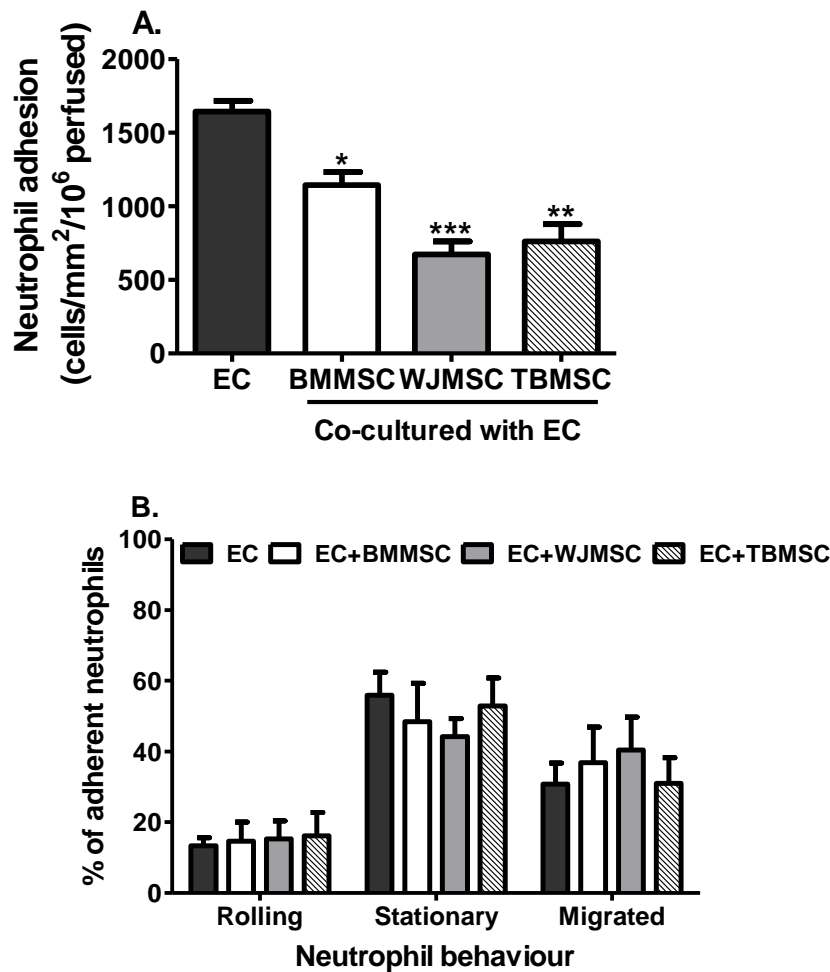
**Figure 4-4: Effect of the number of WJMSC seeded onto filters on neutrophil recruitment to TNF $\alpha$  treated EC in a filter flow based adhesion assay.** WJMSC were seeded at different numbers ( $1 \times 10^5$ ,  $3 \times 10^5$  or  $5 \times 10^5$ ) onto filters and co-cultured with EC for 24h. Parallel EC mono-cultures were also set up. Cultures were stimulated with 100U/ml TNF $\alpha$  for 4h and neutrophil (A) adhesion and (B) behaviour were analysed. Neutrophil behaviour was expressed as the percentage of adherent neutrophils that were rolling, stationary and migrated. Data are mean  $\pm$  SEM from n= 5 experiments. In A, ANOVA showed a significant effect of culture conditions on neutrophil adhesion. \* =  $p < 0.05$  and \*\* =  $p < 0.01$  compared to EC mono-cultures by Dunnett post-test. In B, ANOVA showed no significant effect of culture conditions on neutrophil behaviour. A different EC, neutrophil and WJMSC donor was used per experiment.

Subsequently we compared the suppressive effect of BMMSC, WJMSC and TBMSC on neutrophil recruitment using the filter-based adhesion assay. Similar to the microchannel model, co-culture with all 3 MSC populations significantly suppressed neutrophil adhesion to EC to a similar extent, compared to EC mono-cultures in response to TNF $\alpha$  (Figure 4-5A). Furthermore, co-culture had no significant effect on the behaviour of the adherent neutrophils (Figure 4-5B). All subsequent adhesion assays were performed using the filter flow based model.

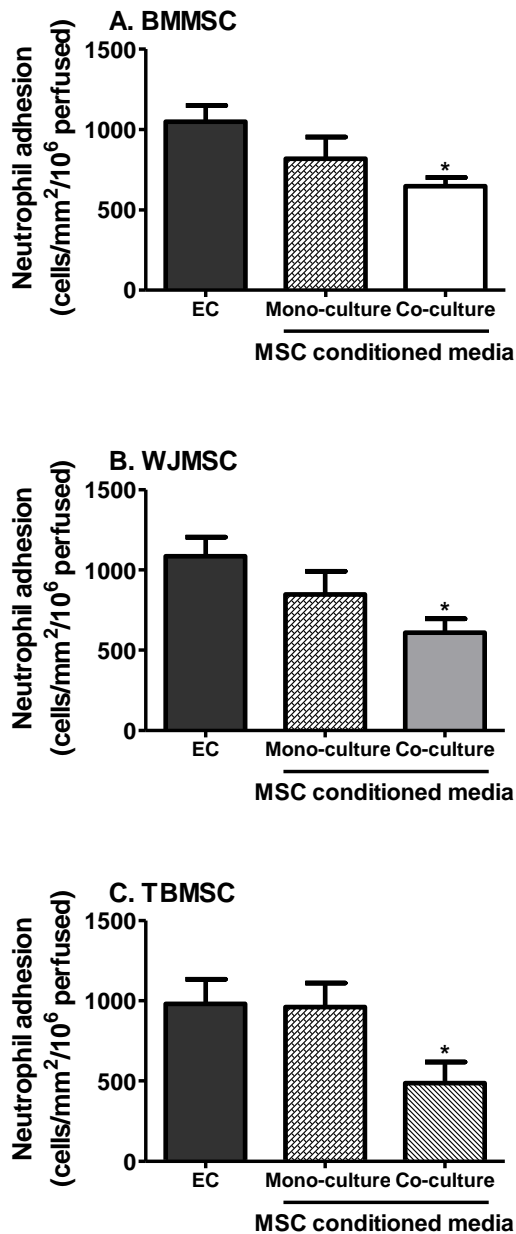
#### *4.2.4. Effect of MSC co-culture conditioned media on neutrophil recruitment*

Next, we examined whether the effects of co-culture were mediated by secreted soluble factors. To test this, EC mono-cultures were treated with conditioned media from BMMSC, WJMSC or TBMSC mono-cultures or co-cultures for 24h prior to TNF $\alpha$ -stimulation for a further 4h in the same culture media. Conditioned media from co-cultures with all MSC populations mimicked the suppressive effects of direct MSC co-culture (Figure 4-6). In contrast, mono-culture conditioned media from all MSC populations was effectively inert (Figure 4-6). In addition, neutrophil behaviour was not significantly affected by any conditioned media tested (Figure 4-7). However, a significant reduction in the percentage of stationary neutrophils was observed when EC were treated with TBMSC mono-culture or co-culture conditioned media (Figure 4-7). Therefore, the immunosuppressive effects of MSC may be attributed to bioactive agent(s) secreted during co-culture with EC, but not secreted by MSC cultured alone. However, as the effect of EC mono-culture conditioned media was not tested, it cannot be ruled out that the suppressive effects of co-culture conditioned media are due to factors secreted by the EC alone.

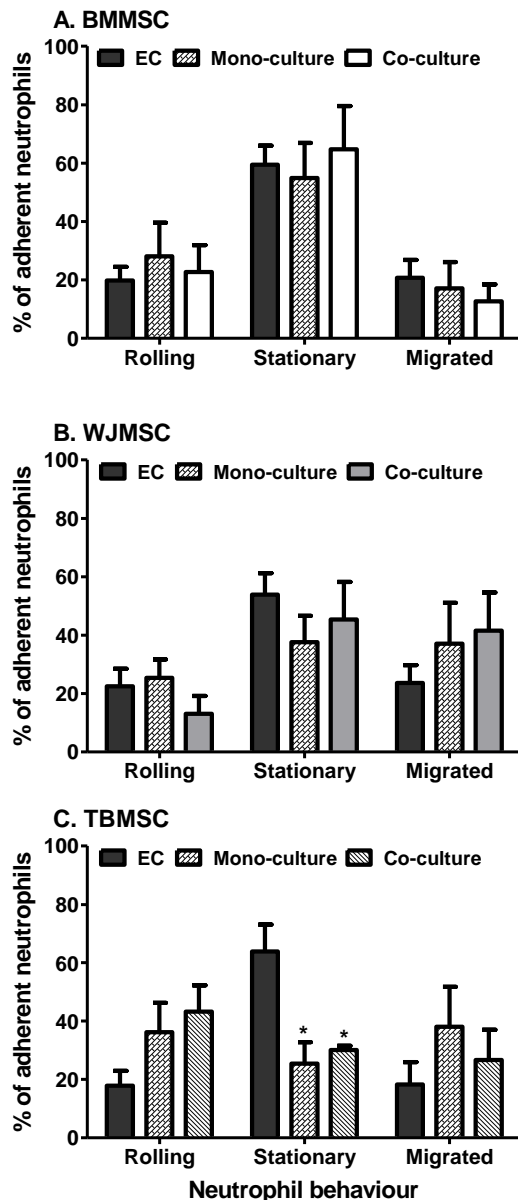




**Figure 4-5: Effect of co-culture of EC with different MSC populations on neutrophil adhesion.** BMMSC, WJMSC and TBMSC ( $5 \times 10^5$ ) were co-cultured with EC on opposite sides of filters for 24h prior to stimulation with 100U/ml TNF $\alpha$  for 4h. Neutrophil (A) adhesion and (B) behaviour were then assessed. Neutrophil behaviour was expressed as the percentage of adherent neutrophils that were rolling, stationary and migrated. Data are mean  $\pm$  SEM from  $n = 5$  experiments. Assays performed with WJMSC co-cultures were carried out on separate days, but EC mono-culture controls were performed on each occasion. Therefore, comparisons to controls were performed using independent paired t-tests. \* =  $p < 0.05$  and \*\* =  $p < 0.01$  compared to EC mono-cultures. In B, ANOVA showed no significant effect of culture conditions on neutrophil behaviour. A different EC and neutrophil donor was used per experiment. 3 different BMMSC and 5 different WJMSC and TBMSC donors were used in this set of experiments.



**Figure 4-6: Effect of conditioned media from MSC cultured alone or with EC on neutrophil adhesion from flow.** Conditioned media was harvested from (A) BMMSC, (B) WJMSC or (C) TBMSC cultured alone or with EC for 24h. EC mono-cultures were treated with conditioned media for 24h before stimulation with 100U/ml TNF $\alpha$  for 4h in the same culture media. Neutrophil adhesion was analysed. Data are mean  $\pm$  SEM from n= 3 experiments. Assays performed with MSC mono-cultures and co-culture conditioned media were carried out on separate days, but EC mono-culture controls were performed on each occasion. Therefore, comparisons to controls were performed using independent paired t-test. \* = p<0.05 compared to EC mono-cultures. A different EC and neutrophil donor was used per experiment. 2 different BMMSC and 3 different WJMSC and TBMSC donors were used in this set of experiments.



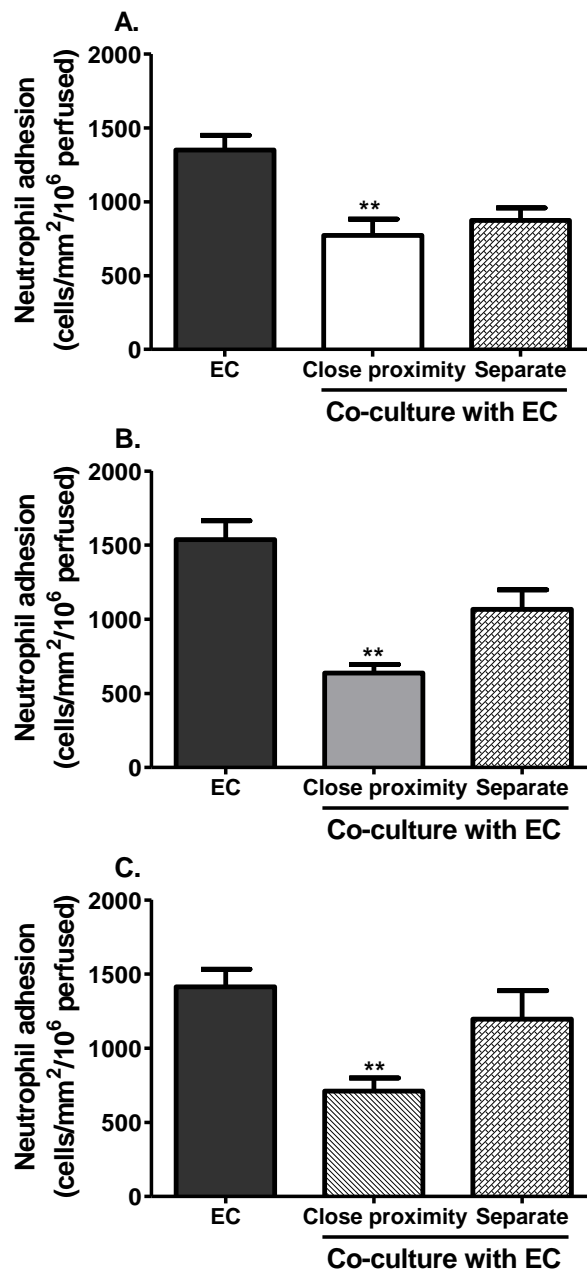
**Figure 4-7: Effect of conditioned media from MSC cultured alone or with EC on neutrophil behaviour.** Conditioned media was harvested from (A) BMMSC, (B) WJMSC or (C) TBMSC cultured alone or with EC for 24h. EC mono-cultures were treated with conditioned media for 24h before stimulation with 100U/ml TNF $\alpha$  for 4h in the same culture media. Neutrophil behaviour was analysed and expressed as the percentage of adherent neutrophils that were rolling, stationary and migrated. Data are mean  $\pm$  SEM from n= 3 experiments. Assays performed with MSC mono-culture and co-culture conditioned media were carried out on separate days, but EC mono-culture controls were performed on each occasion. In A and B, ANOVA showed no significant effect of MSC conditioned media on neutrophil behaviour. In C, ANOVA showed a significant effect of TBMSC co-culture on neutrophil behaviour,  $p < 0.05$ . \* =  $p < 0.05$  compared to EC mono-cultures by Bonferroni post-test. A different EC and neutrophil donor was used per experiment. 2 different BMMSC and 3 different WJMSC and TBMSC donors were used in this set of experiments.

#### *4.2.5. Effect of proximity between MSC and EC on neutrophil recruitment*

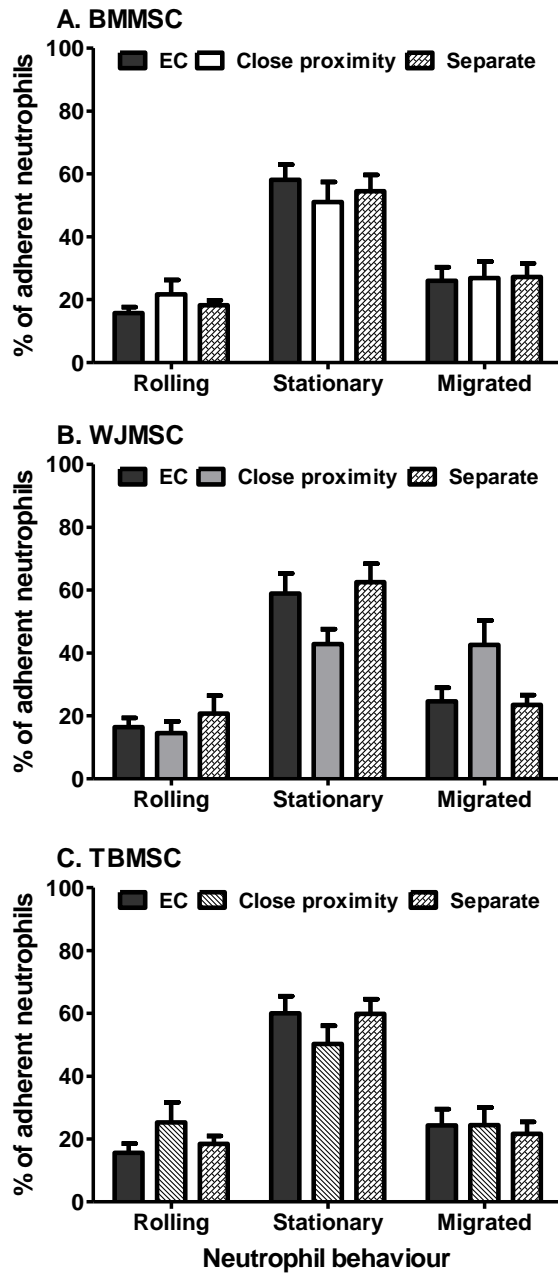
Next, we examined whether close proximity between MSC and EC was required for limiting neutrophil adhesion. MSC were seeded onto a 6-well plate while EC were cultured above on a Transwell filter (in the same well) for 24h at a distance of 0.9mm. The suppressive effect of BMMSC on neutrophil adhesion was only slightly reduced when separated from EC compared to co-cultures on opposite sides of the filter, although the suppression was no longer statistically significant (Figure 4-8A). In contrast, separation of WJMSC or TBMSC from EC reduced their capacity to suppress neutrophil adhesion (Figure 4-8B and C). The behaviour of adherent neutrophils was not significantly altered by separation of EC from all MSC populations (Figure 4-9). Therefore, MSC from different sources differ in their requirement for proximity to EC to generate the bioactive agent(s) that mediate their functional effects on recruitment.

#### *4.2.6. Role of IL-6 and TGF $\beta$ in the suppression of neutrophil recruitment by MSC from different sources*

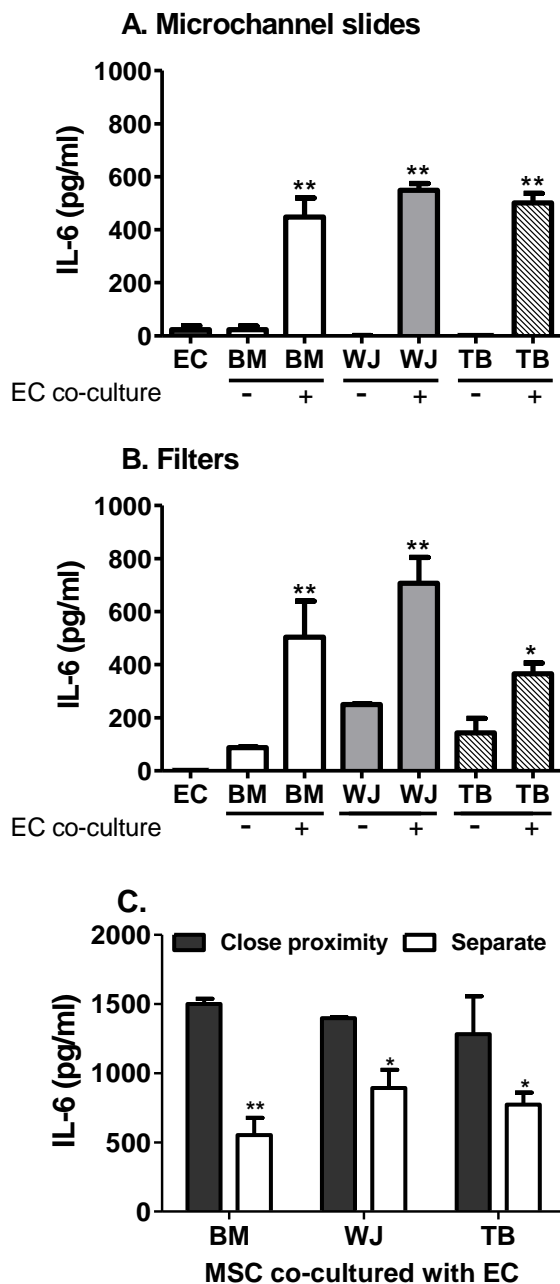
IL-6 secretion was previously found to be upregulated in BMMSC co-cultures with EC when both cell types were in direct contact in microslide channels (Luu *et al.*, 2013). Indeed, the immunosuppressive effects of BMMSC were found to be mediated by IL-6 and TGF $\beta$  (Luu *et al.*, 2013). We hypothesised that IL-6 may be a common bioactive agent mediating suppression by all MSC populations. To examine this, we quantified IL-6 levels in supernatants from 24h EC, BMMSC, WJMSC and TBMSC mono-cultures and co-cultures. IL-6 secretion was significantly increased in co-culture with all MSC populations when MSC were incorporated into the EC monolayer (Figure 4-10A) or on the opposite sides of a Transwell filter (Figure 4-10B) compared to EC mono-culture.



**Figure 4-8: Effect of varying closeness of contact between EC and MSC during co-culture on suppression of neutrophil adhesion.** Co-cultures were formed by seeding (A) BMMSC, (B) WJMSC or (C) TBMSC on the opposite side of Transwell filters to EC (Close proximity) or by seeding MSC on the 6-well plate below and EC above on a filter (Separate). Cultures were treated with 100U/ml TNF $\alpha$  for 4h and neutrophil adhesion was analysed. Data are mean  $\pm$  SEM from n= 3-13 experiments. Close proximity and Separate culture assays were carried out on separate days, but EC mono-culture controls were performed on each occasion. Therefore, comparisons to controls were performed using independent paired t-test. \*\* = p<0.01 compared to EC mono-cultures. A different EC and neutrophil donor was used per experiment. 2 different BMMSC and at least 3 different WJMSC and TBMSC donors were used in this set of experiments.



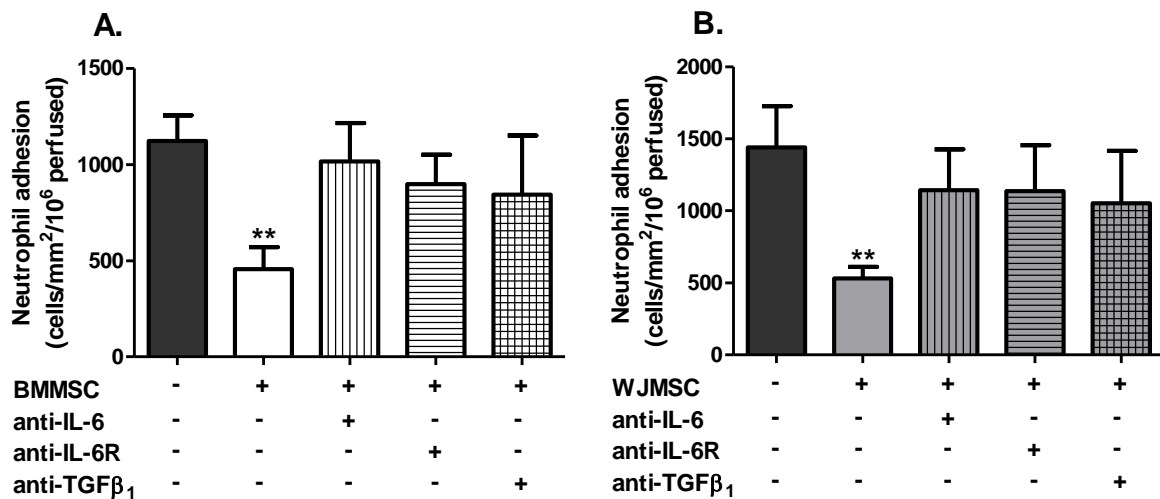
**Figure 4-9: Effect of varying closeness of contact between EC and MSC during co-culture on neutrophil behaviour.** Co-cultures were formed by seeding (A) BMMSC, (B) WJMSC or (C) TBMSC on the opposite side of Transwell filters to EC (Close proximity) or by seeding MSC on the 6-well plate below and EC above on a filter (Separate). Cultures were treated with 100U/ml TNF $\alpha$  for 4h. Neutrophil behaviour was analysed and expressed as the percentage of adherent neutrophils that were rolling, stationary and migrated. Data are mean  $\pm$  SEM from n= 3-13 experiments. Close proximity and separate culture assays were carried out on separate days, but EC mono-culture controls were performed on each occasion. Paired t-test showed no significant effect of MSC conditioned media treatment on neutrophil behaviour. A different EC and neutrophil donor was used per experiment. 2 different BMMSC and at least 3 different WJMSC and TBMSC donors were used in this set of experiments.



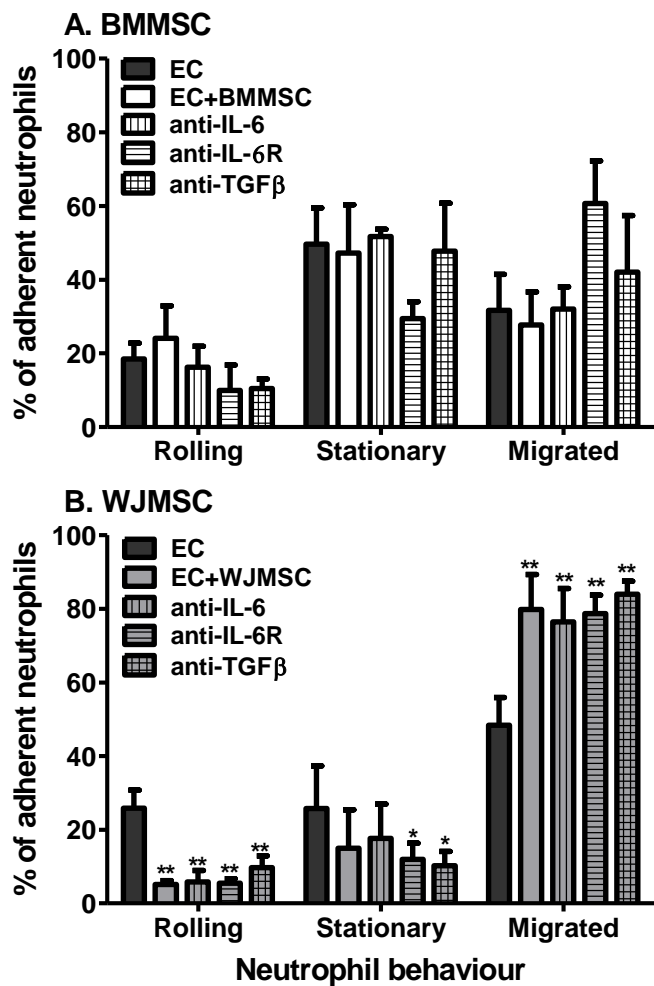
**Figure 4-10: Levels of IL-6 in EC and MSC mono-culture and co-culture supernatants cultured in varying degrees of closeness.** IL-6 was measured in supernatants from mono-cultures and co-cultures of EC and all MSC populations from (A) microchannel slides, (B) filters and (C) co-cultures formed on opposite sides of the filter (Close proximity) or in the absence of contact (Separate). Data are mean  $\pm$  SEM from  $n=3$  independent experiments. In B, WJMSC mono-culture supernatants were tested on a separate day to the other conditions, but EC mono-culture controls were performed on each occasion. In A, B and C, ANOVA showed a significant effect of culture conditions on IL-6 secretion,  $p<0.01$ . \* =  $p<0.05$  and \*\* =  $p<0.01$  compared to EC mono-cultures by Dunnett post-test.

In addition, the levels generated during co-culture tended to be greater than the sum of the EC and MSC mono-cultures (Figure 4-10A and B). Separating EC from BMMSC, WJMSC and TBMSC significantly reduced IL-6 secretion compared with co-cultures on opposite sides of a filter (Figure 4-10C). Therefore, IL-6 may be a common soluble mediator in EC co-cultures with all MSC populations. To assess whether IL-6 and TGF $\beta$  played a role in suppression of neutrophil adhesion by MSC from different sources, BMMSC or WJMSC were cultured with EC, in the presence or absence of antibodies targeting soluble IL-6, IL-6R or TGF $\beta$  for the duration of the co-culture and cytokine treatment. In this series of experiments, p4 WJMSC were used as there were too few p3 WJMSC available. Additionally, TBMSC were not examined as there were too few cells available to perform these assays. BMMSC and WJMSC were found to significantly suppress neutrophil recruitment compared to EC alone (Figure 4-11). Inhibition of IL-6 (by neutralising IL-6 or blocking IL-6R) or TGF $\beta$  signalling during co-culture abrogated suppression of neutrophil adhesion by BMMSC or WJMSC (Figure 4-11). Blocking IL-6 or TGF $\beta$  signalling had no significant effect on the behaviour of adherent neutrophils (Figure 4-12). Co-culture of EC with WJMSC significantly increased neutrophil transmigration, but this effect was not significantly altered by inhibiting IL-6 or TGF $\beta$  signalling (Figure 4-12B). Increased neutrophil migration in p4 WJMSC co-cultures was concomitant with a significant reduction in the percentage of rolling and stationary neutrophils (Figure 4-12B). This suggests that co-culture with later passage WJMSC may promote migration. Collectively, these data indicate that IL-6 and TGF $\beta$  are drivers of BMMSC and WJMSC-mediated suppression of neutrophil recruitment.





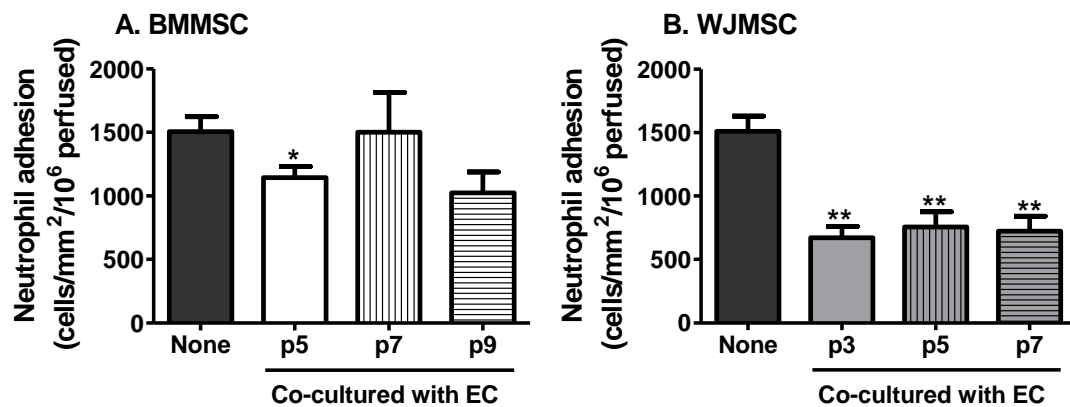
**Figure 4-11: Effect of blocking actions of IL-6 or TGFβ on the capacity of MSC in co-culture to suppress neutrophil adhesion.** (A) BMMSC or (B) WJMSC co-cultures were treated with neutralising antibodies against IL-6 or TGFβ, or a function blocking antibody against IL-6R for the duration of the co-culture and cytokine treatment. Neutrophil adhesion was assessed. Data are mean ± SEM from n= 4 experiments. ANOVA showed a significant effect of culture conditions on neutrophil adhesion,  $p < 0.01$ . \*\* =  $p < 0.01$  compared to EC mono-cultures by Dunnett post-test. A different EC and neutrophil donor was used per experiment. 3 different BMMSC and 4 different WJMSC donors were used in this set of experiments.



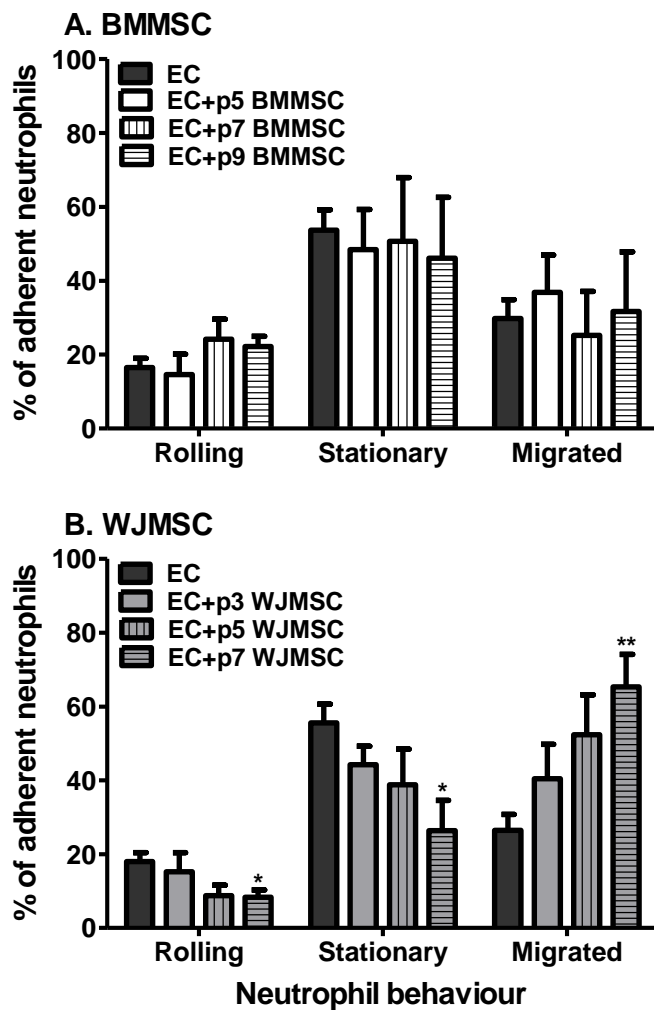
**Figure 4-12: Effect of blocking actions of IL-6 or TGFβ in MSC co-cultures on neutrophil behaviour.** (A) BMMSC or (B) WJMSC co-cultures were treated with neutralising antibodies against IL-6 or TGFβ, or a function blocking antibody against IL-6R for the duration of the co-culture and cytokine treatment. Neutrophil behaviour was analysed and expressed as the percentage of adherent neutrophils that were rolling, stationary and migrated. Data are mean ± SEM, n= 4 experiments. In A, ANOVA showed no significant effect of culture conditions on neutrophil behaviour. In B, ANOVA showed a significant effect of culture conditions on neutrophil behaviour,  $p < 0.01$ . \* =  $p < 0.05$  and \*\* =  $p < 0.01$  compared to EC mono-cultures by Bonferroni post-test. A different EC and neutrophil donor was used per experiment. 3 different BMMSC and 4 different WJMSC donors were used in this set of experiments.

#### *4.2.7. Effects of passage on MSC-mediated suppression of neutrophil recruitment*

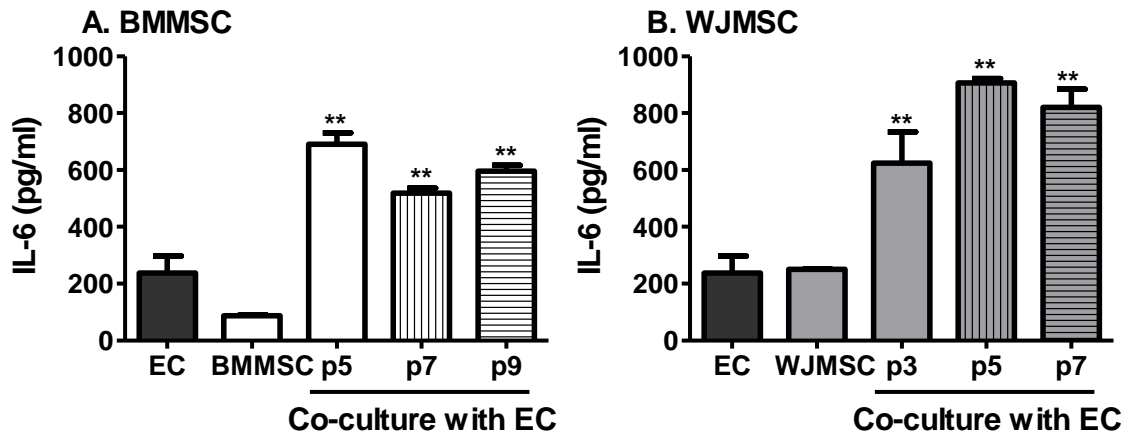
To determine whether passaging MSC altered their ability to suppress neutrophil recruitment, the effect of EC co-culture with p5, p7 and p9 BMMSC or p3, p5 and p7 WJMSC was tested. TBMSC were not examined as they could not be expanded beyond p3. p5 BMMSC were found to significantly suppress neutrophil adhesion and this effect was diminished at p7 and p9 (Figure 4-13A). WJMSC retained their ability to significantly suppress neutrophil adhesion up to p7 (Figure 4-13B). Increasing passage of BMMSC to p7 and p9 did not significantly alter the behaviour of neutrophils adherent to co-cultures (Figure 4-14A). In contrast, increasing passage of WJMSC tended to reduce the percentage of rolling and stationary neutrophils and increase the percentage migrated compared to EC mono-cultures (Figure 4-14B). However, the change in neutrophil behaviour was only significant at p7 WJMSC co-culture (Figure 4-14B). Next, we measured IL-6 generated by BMMSC and WJMSC co-cultures over passage to assess whether the loss of immunosuppression by MSC at later passage (Figure 4-13) was due to a reduction in IL-6 secretion in co-culture. However, co-cultures at all passages of BMMSC and WJMSC produced similar levels of IL-6 (Figure 4-15). Furthermore, the levels of IL-6 in co-culture were significantly higher than the individual mono-cultures (Figure 4-15). This suggests that while IL-6 may have been bioactive in co-culture, it was not the sole mediator responsible for the immunosuppression by these MSC populations.



**Figure 4-13: Effect of BMMSC and WJMSC passage on their ability to suppress neutrophil adhesion.** EC were co-cultured with (A) BMMSC or (B) WJMSC at different passage, on opposite sides of a filter for 24h prior to stimulation with TNF $\alpha$  for 4h. Parallel EC mono-cultures were set up and neutrophil adhesion was assessed. Data are mean  $\pm$  SEM from n= 3-5 experiments. Assays at each passage were carried out on separate days, but EC mono-culture controls were performed on each occasion. Therefore, comparisons to controls were performed using an unpaired t-test. \* = p<0.05 and \*\* = p<0.01 compared to EC mono-cultures. A different EC and neutrophil donor was used per experiment. At least 2 different BMMSC and 3 different WJMSC donors were used in this set of experiments.



**Figure 4-14: Effect of BMMSC and WJMSC passage on the behaviour of neutrophils adherent to EC co-cultured with MSC.** EC were co-cultured with (A) BMMSC or (B) WJMSC at different passage, on opposite sides of a filter for 24h prior to stimulation with TNF $\alpha$  for 4h. Parallel EC mono-cultures were set up and the behaviour of adherent neutrophils was assessed. Neutrophil behaviour was expressed as the percentage of adherent neutrophils that were rolling, stationary and migrated. Data are mean  $\pm$  SEM from n= 3-5 experiments. Assays at each passage were carried out on separate days, but EC mono-culture controls were performed on each occasion. Therefore, in B, comparisons to controls were performed using independent paired t-tests. \* = p<0.05 and \*\* = p<0.01 compared to EC mono-cultures. A different EC and neutrophil donor was used per experiment. At least 2 different BMMSC and 3 different WJMSC donors were used in this set of experiments.



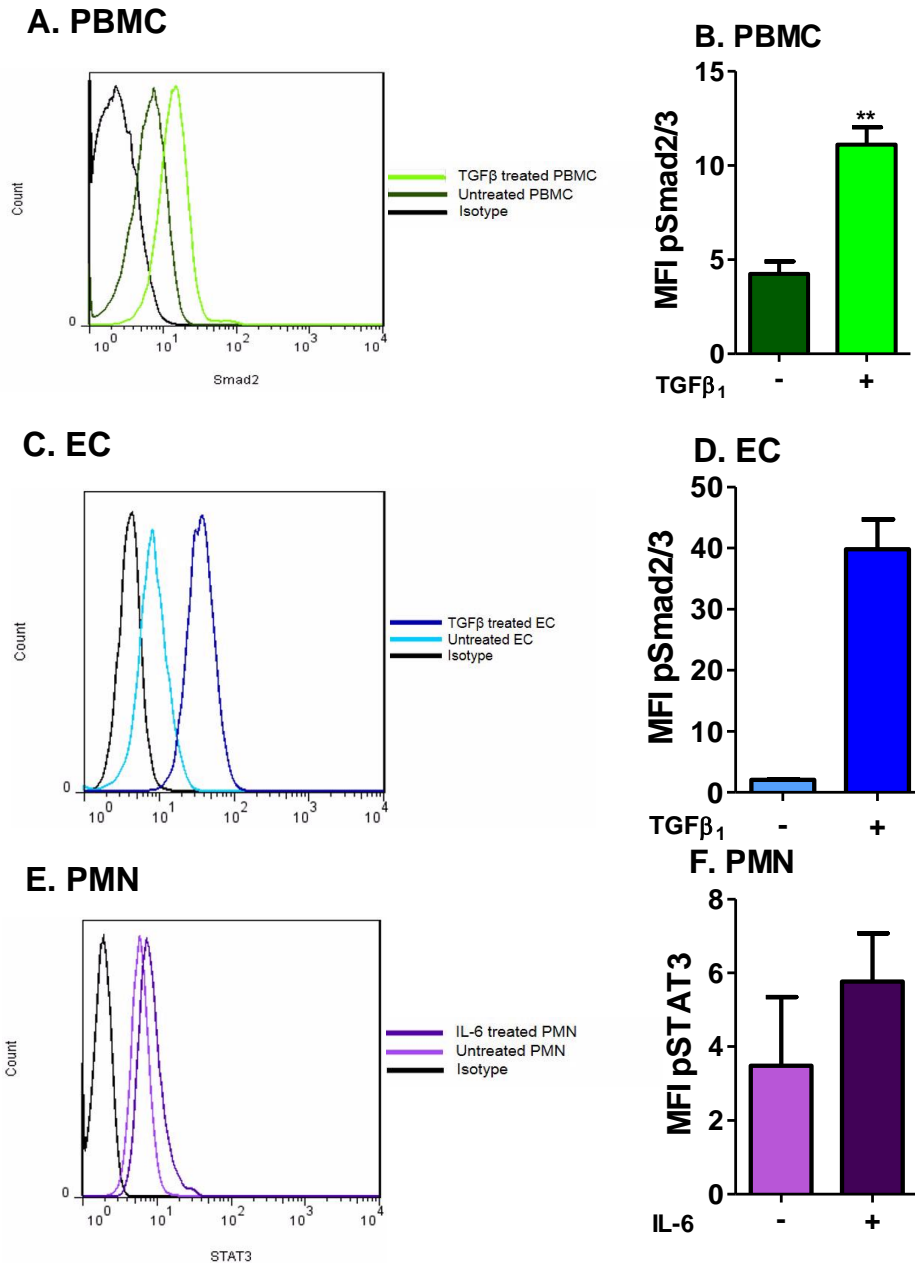
**Figure 4-15: Effect of MSC passage on the levels of IL-6 in EC and MSC mono-culture and co-culture supernatants.** IL-6 was measured in supernatants from mono-cultures or co-cultures of EC with (A) BMMSC, or (B) WJMSC at different passage. Data are mean  $\pm$  SEM from  $n=3$  experiments. In A and B, ANOVA showed a significant effect of culture conditions on IL-6 secretion,  $p<0.01$ . \*\* =  $p\leq 0.01$  compared to EC mono-cultures by Dunnett post-test.

#### *4.2.8. Assessment of downstream IL-6 and TGF $\beta$ signalling during co-culture*

To identify whether EC and/or MSC in the co-culture responded to soluble IL-6 and TGF $\beta$ , we set out to measure internal phosphorylated STAT3 (pSTAT3; downstream of IL-6) and phosphorylated Smad2/3 (pSmad2/3; downstream of TGF $\beta$ ) in the BMMSC and EC during co-culture by flow cytometry. Initially, we validated the anti-pSmad2/3 antibody by treating PBMC with recombinant TGF $\beta$  and then antibody staining the cells (as described in Section 2.7.2.). Treatment of PBMC with recombinant TGF $\beta_1$  increased the levels of pSmad2/3 (Figure 4-16A-B) confirming that the antibody could detect intracellular TGF $\beta$  signalling.

We next assessed the expression of pSmad2/3 by EC after treatment with recombinant TGF $\beta_1$  to determine whether EC are able to respond to TGF $\beta$  and upregulate intracellular pSmad2/3. Expression of pSmad2/3 was upregulated by TGF $\beta$  treated EC compared to untreated EC (Figure 4-16C-D) indicating that the cells express the corresponding receptors and are able to signal through pSmad2/3.

To validate the pSTAT3 antibody, PMN were treated with recombinant IL-6 and stained with anti-pSTAT3. While this cell type is known to respond to IL-6 and increase phosphorylation of STAT3, in our hands treatment with IL-6 did not increase the levels of pSTAT3 above the isotype control (Figure 4-16E-F). As IL-6 induced STAT3 phosphorylation and degradation occurs rapidly, identifying the exact time at which this process occurs is very difficult. We attempted to measure pSTAT3 in IL-6 treated PMN at several earlier time points but once again pSTAT3 could not be detected (n=1). Consequently, pSTAT3 was not measured in co-cultures.

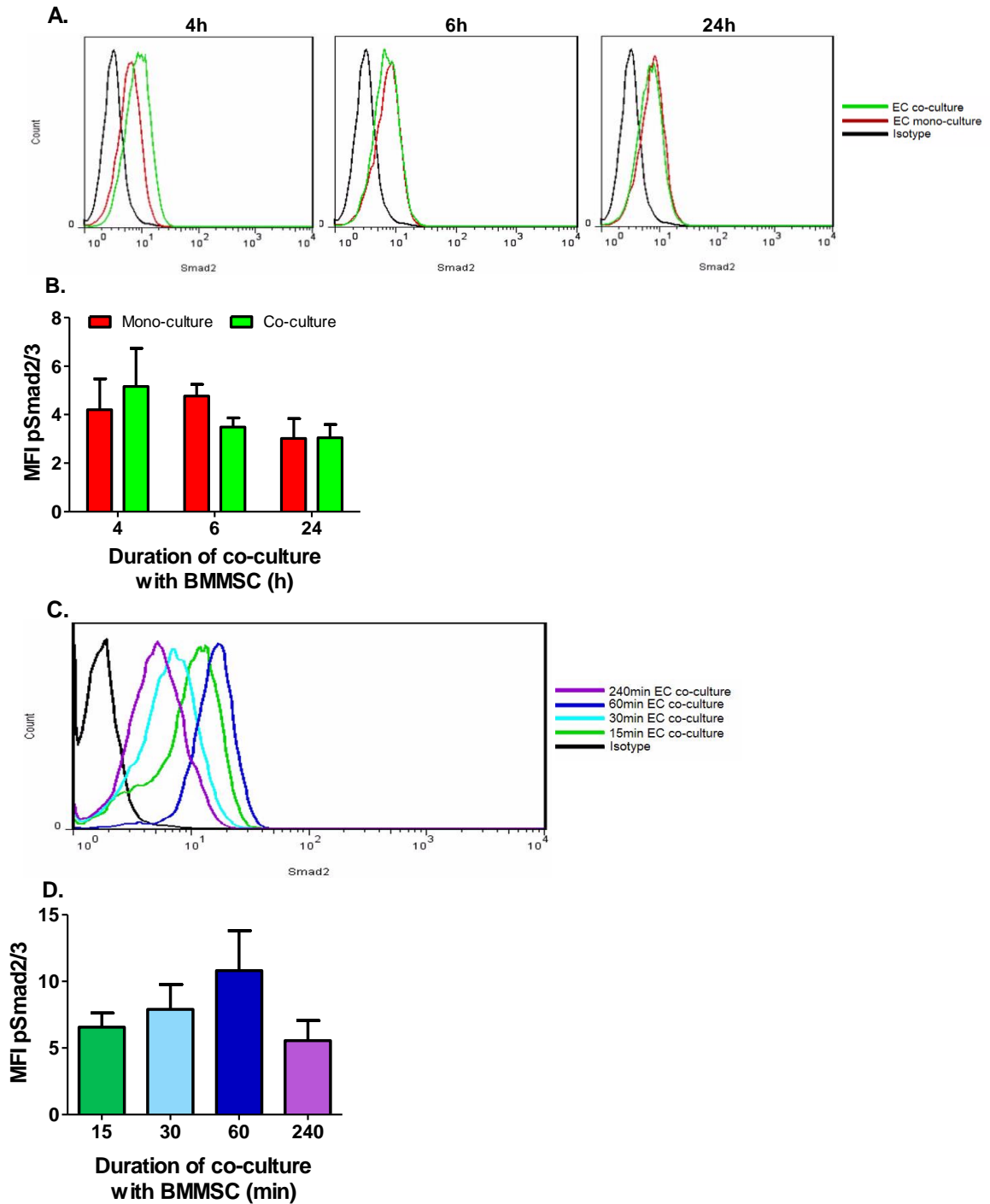


**Figure 4-16: Analysis of phosphorylated Smad2/3 or STAT3 in leukocytes or EC treated with TGFβ and IL-6.** (A-D) PBMC or EC were treated with recombinant TGFβ<sub>1</sub> for 30min and stained with anti-pSmad2/3. In C and D, untreated EC mono-cultures were set up as a control. (A,C) Representative histogram plots of pSmad2/3 stained PBMC or EC compared to isotype control. (B,D) Median fluorescence intensity (MFI) for pSmad2/3 in TGFβ treated PBMC or EC after subtracting the isotype MFI. (E,F) PMN were treated with IL-6 for 15min and stained with anti-pSTAT3. (E) Representative histogram plot of pSTAT3 stained PMN. (F) MFI for pSTAT3 in IL-6 treated PMN after subtracting the isotype MFI. Data are mean ± SEM from n= 3 experiments. In B, paired t-test showed a significant effect of TGFβ-stimulation of Smad2/3 expression by PBMC; \*\* = p<0.01. In D and F, paired t-test showed no significant effect of treatment on expression. A different PBMC, EC and PMN donor was used per experiment.

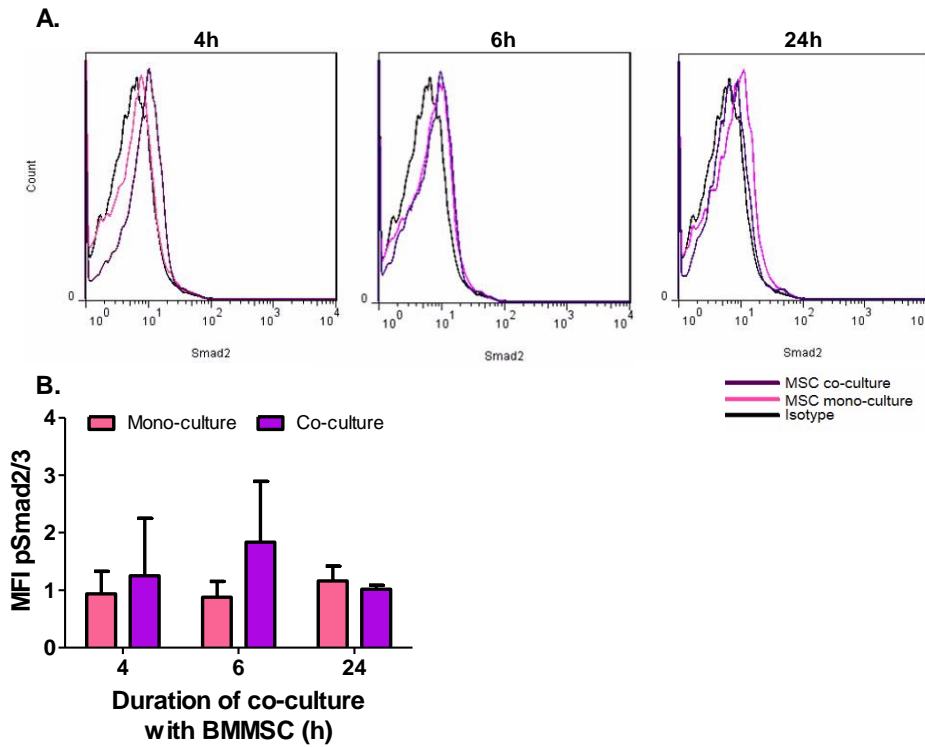


We subsequently assessed whether pSmad2/3 was upregulated in EC during co-culture and how early in the co-culture this signalling occurred. pSmad2/3 expression in EC co-cultures was measured at 4h, 6h and 24h and compared to EC mono-cultures. Levels of pSmad2/3 was comparable in the EC mono-cultures and co-cultures at each time point (Figure 4-17A-B). Furthermore, expression of pSmad2/3 in all cultures did not change over time (Figure 4-17A-B).

We then assessed pSmad2/3 levels in EC at earlier time points (15min, 30min, 60min and 240min) during co-culture, in case we missed its upregulation. A steady increase in pSmad2/3 levels was observed between 15min and 60min which was then reduced at 240min, although the effect was not significant (Figure 4-17C-D). Furthermore, the median fluorescence intensity (MFI) values for pSmad2/3 were very low compared to TGF $\beta$  treated EC mono-cultures (Figure 4-16D) indicating that TGF $\beta$  downstream signalling in EC was not significantly upregulated at any point during co-culture. Analysis of pSmad2/3 in BMMSC mono-cultures and co-cultures at 4h, 6h and 24h also showed that co-culture had no effect on pSmad2/3 levels in BMMSC compared to mono-cultures (Figure 4-18). The MFI values for pSmad2/3 were very low and were comparable to the isotype control (Figure 4-18). pSmad2/3 levels in BMMSC at earlier time points during co-culture were not assessed as there were not enough cells available to perform these analyses. Collectively, these data suggest that co-culture does not affect intracellular TGF $\beta$  signalling in either EC or BMMSC in co-culture compared to mono-cultures.



**Figure 4-17: Analysis of pSmad2/3 in EC during co-culture with BMMSC.** EC were seeded onto filters with BMMSC for (A,B) 4h, 6h and 24h or (C,D) 15min, 30min, 60min and 240min, then detached and stained for pSmad2/3. In A and B, parallel EC mono-cultures were also set up. (A,C) Representative histogram plots of pSmad2/3 stained EC at each time point compared to isotype control. (B,D) MFI for pSmad2/3 in EC after different lengths of time in co-culture after subtracting the isotype MFI. Data are mean  $\pm$  SEM from n=3 experiments. A different EC and BMMSC donor was used per experiment.



**Figure 4-18: Analysis of pSmad2/3 in BMMSC during co-culture with EC.** EC were seeded onto filters with BMMSC for 4h, 6h and 24h, BMMSC were then detached and stained for pSmad2/3. Parallel BMMSC mono-cultures were also set up. (A) Representative histogram plots of pSmad2/3 stained BMMSC at each time point compared to isotype control. (B) MFI for pSmad2/3 in BMMSC after different lengths of time in co-culture after subtracting the isotype MFI. Data are mean  $\pm$  SEM from n=3 experiments. A different EC and BMMSC donor was used per experiment.

### 4.3. Discussion

In this chapter we utilised two *in vitro* flow based adhesion models with varying degrees of proximity between the MSC and EC to compare the ability of MSC from different tissues to regulate leukocyte recruitment. We observed that BMMSC, WJMSC and TBMSC suppressed the EC response to cytokine stimulation and limited neutrophil adhesion. Whilst all MSC types exerted the same effect on neutrophil adhesion, we observed several differences between the populations. WJMSC retained their immunosuppressive effects with passage while the effects of BMMSC was diminished by passage.

In addition, WJMSC and TBMSC required close proximity with the EC to induce suppression while BMMSC could exert their effects at a distance albeit to a lesser degree. Co-culture conditioned media from all MSC populations suppressed neutrophil adhesion and IL-6 was found to be a common bioactive agent. However, higher passage BMMSC co-cultures still secreted high levels of IL-6 despite losing their suppressive effects on neutrophil recruitment, indicating a role for other bioactive agent(s). We previously showed that TGF $\beta$  was also a key regulator in BMMSC-mediated suppression of neutrophil recruitment (Luu *et al.*, 2013). Here, neutralisation of IL-6 and TGF $\beta$  effectively inhibited suppression by BMMSC and WJMSC in co-culture showing that concerted action of both mediators was required to suppress neutrophil recruitment for either MSC population.

While all MSC populations had similar effects on neutrophil adhesion, in the filter model later passage WJMSC were found to promote neutrophil migration compared to

BMMSC and TBMSC. This effect of WJMSC in co-culture with EC has not been previously reported. While the mechanism mediating this effect was not examined in this thesis, we hypothesise that it may have been driven by chemokines released by the WJMSC which bound to the EC surface to promote neutrophil migration. To examine the role of chemokines in WJMSC co-cultures, expression of IL-8 should be measured as it is a key mediator that drives neutrophil migration (Imaizumi *et al.*, 1997).

The behaviour of adherent neutrophils was unaffected by co-culture with BMMSC and TBMSC which disagrees with our previous study which showed that BMMSC suppressed migration when incorporated in the EC monolayer (Luu *et al.*, 2013). The discrepancies in the findings may be due to differences in the potency of the TNF $\alpha$  used as they were both purchased from different suppliers. The TNF $\alpha$  batch used in this study was found to be more potent than the batch used in the previous study. Indeed, using the microslide channel model, the percentage neutrophil migration in this study was found to be 40-60% on EC alone compared to 30-35% in the previous report (Luu *et al.*, 2013). High concentrations of TNF $\alpha$  (100U/ml) used in this study may have overcome the suppressive effects of MSC on migration.

MSC can mediate their suppressive effects through the release of soluble factors such as IDO and PGE<sub>2</sub>, with efficacy enhanced by direct contact with target cells [reviewed by (Munir and McGettrick, 2015)]. To test whether the MSC-mediated suppression was through released agents, we cultured EC in MSC conditioned media for the same length of time as the standard co-culture. Mono-culture conditioned media from all

MSC populations were inert while their suppressive effects were transferable in the co-culture conditioned media. These results suggest that, similar to other immune-protective responses of MSC, suppression of neutrophil adhesion by MSC is mediated through soluble agent(s). However, in this case, the factor(s) were only generated when MSC from the different sources were co-cultured with EC. This is consistent with our previous study (Luu *et al.*, 2013) with BMMSC alone. In another report, medium from pulmonary EC and BMMSC co-cultures, but not mono-cultures, reduced adhesion of U937 cells (a monocytic leukaemia cell line) to TNF $\alpha$ -treated pulmonary EC under static conditions (Pati *et al.*, 2011). This suggests that crosstalk between MSC and EC is essential for mediating suppression of neutrophil recruitment during inflammation.

As the soluble factors responsible for mediating suppression were only generated during co-culture with EC, we subsequently tested whether close proximity between the MSC and EC was required for inducing crosstalk. EC and MSC cultured on opposite sides of a filter were held ~10 $\mu$ m apart, but cell-cell interactions may have occurred via the tips of protruding pseudopods through 0.4 $\mu$ m pores in the filter. Separation of MSC from EC by ~900 $\mu$ m significantly impaired the suppressive effects of WJMSC and TBMSC to a greater extent than BMMSC. This may have been due to differences in the sensitivity of MSC from different tissue sources to factor(s) released by EC; with WJMSC and TBMSC having a lower sensitivity than BMMSC. Of note, separation of the two cell types in these assays may not represent the *in vivo* situation as generally the distance between tissue-resident cells and local blood vessels are unlikely to be as far apart as the distance between MSC and EC in these assays. Thus, *in vivo*, WJMSC and TBMSC may only be able to secrete soluble factor(s) mediating suppression of neutrophil infiltration when in close proximity to vascular EC.

Interestingly, IL-6 secretion was significantly reduced in separated WJMSC and TBMSC co-cultures suggesting that the soluble mediator(s) required to induce their immunosuppressive effect were only generated to sufficient levels when cells were in close proximity.

Consistent with our previous report (Luu *et al.*, 2013), crosstalk between EC and BM MSC, WJMSC or TB MSC was required to produce the high levels of IL-6 present in co-culture conditioned media. While IL-6 is often described as an inflammatory cytokine, that inhibits expansion of T<sub>reg</sub> cell populations and activated T-cell apoptosis [reviewed by (Scheller *et al.*, 2011)], recent studies have shown that it acts on EC in a context dependent manner (Lally *et al.*, 2005; Kuravi *et al.*, 2014; Luu *et al.*, 2013; McGettrick *et al.*, 2009). In published studies, IL-6 either alone or in conjunction with TGF $\beta$  modified inflammatory infiltrates by inhibiting recruitment (Ulich *et al.*, 1991; McGettrick *et al.*, 2009) or by switching recruitment from neutrophils to mononuclear leukocytes (Hurst *et al.*, 2001; Kaplanski *et al.*, 2003; Rose-John *et al.*, 2007). Furthermore, in our laboratory, pre- treating cytokine-stimulated EC with recombinant IL-6 suppressed recruitment of PBL or neutrophils suggesting that it can also act in an anti-inflammatory manner (Butler *et al.*, 2011; McGettrick *et al.*, 2009). Thus, in the context of leukocyte recruitment during inflammation, IL-6 appears to act in an anti-inflammatory manner.

We have previously shown that TGF $\beta$  is also active in BM MSC-mediated suppression of neutrophil recruitment (Luu *et al.*, 2013). Here, we observed that neutralising either IL-6 or TGF $\beta$  signalling abolished the suppression of neutrophil adhesion by BM MSC

and WJMSC. This suggests that IL-6 and TGF $\beta$  act in conjunction to mediate suppression with one factor likely regulating the signalling of the other, as loss of either factor abrogated the effect. These data suggest that MSC from different tissue sources utilised the same mechanism to mediate suppression of neutrophil adhesion. This was not restricted to leukocyte recruitment, as a previous study showed that BMMSC, WJMSC and ADMSC utilised the same mechanism to suppress proliferation of PHA-induced allogeneic T-cell proliferation and secretion of TNF $\alpha$  and IFN $\gamma$  (Yoo *et al.*, 2009). The effect was mediated by soluble agents that were found to be secreted to similar levels by all 3 MSC populations (Yoo *et al.*, 2009).

*In vitro* expansion can have adverse effects on the proliferation and differentiation potential of adult MSC, which have been found to lose their capacity by p5-7 (Lo Surdo and Bauer, 2012; Choudhery *et al.*, 2014), compared to foetal and neonatal MSC which retain their potential for longer in culture (p12-18) (Giuliani *et al.*, 2011). As well as their proliferation and differentiation capacity, previous studies have shown that the immunomodulatory effects of MSC were altered by passage with foetal and neonatal MSC retaining their suppressive effects (predominantly on T-cell proliferation) for longer in culture than BMMSC (Li *et al.*, 2012; Giuliani *et al.*, 2011; Yu *et al.*, 2014; Zhuang *et al.*, 2014). In agreement, we showed that BMMSC co-cultures lost their ability to inhibit neutrophil adhesion more rapidly with passage than WJMSC. We hypothesised that the loss of suppression was due to a decrease in IL-6 secretion during co-culture. However, we observed that IL-6 levels were consistently higher in co-culture than EC mono-cultures. Therefore, MSC-mediated suppression required the action of other soluble factor(s) that were presumably downregulated by passage. For instance TGF $\beta$  production in co-culture may be reduced by MSC passage which would



abrogate their immunosuppressive capacity. However, the effects of passage on TGF $\beta$  levels in co-culture were not measured.

To test which cells IL-6 and TGF $\beta$  signalled through in the co-culture, we attempted to measure the levels of phosphorylated downstream signalling molecules for the two pathways (pSTAT3 and pSmad2/3 respectively). However, we were unable to detect changes in pSTAT3 in positive control samples. As IL-6-STAT3 signalling is a rapid process that occurs shortly after binding of IL-6 to its receptor, the time point used in these assays (after 30min stimulation with IL-6) may have been too late. Given the rapid kinetics of IL-6-STAT3 signalling, identifying when IL-6 is upregulated in co-culture could provide insight into when its signalling occurs. In future studies IL-6 levels in conditioned media from EC and MSC mono-cultures and co-cultures should be measured at several time points during the co-culture (1h, 4h, 6h, 12h and 24h) to elucidate when IL-6 signalling may occur.

The cell types in co-culture that responded to TGF $\beta$  could not be determined as co-culture did not appear to alter levels of pSmad2/3 in EC or BMMSC. The levels of TGF $\beta$  protein should be quantified in conditioned media to determine the effects of co-culture on its signalling. However, TGF $\beta$  is only active after proteolytic cleavage (Taylor, 2009), therefore, only levels of the active form should be measured in co-culture conditioned media, not the total levels of TGF $\beta$  present. However, levels of active TGF $\beta$  protein in culture supernatants cannot be accurately assessed. Quantitative ELISA kits for measuring TGF $\beta$  have an acid activation step which activates all latent TGF $\beta$  present in culture media. As FCS contains high levels of latent TGF $\beta$ , this also

becomes activated. As a result, the levels of active TGF $\beta$  induced by co-culture would be masked by the levels of FCS-derived TGF $\beta$ . In future studies co-cultures should be set up in serum free culture media and after ensuring that they retain their suppressive effects in the new culture environment, the levels of active TGF $\beta$  should be measured.

Currently, the majority of studies looking at the therapeutic potential of MSC are testing BMMSC, however other groups have shown that proliferation capacity of BMMSC is reduced in culture (Lo Surdo and Bauer, 2012; Choudhery *et al.*, 2014). Furthermore, isolation of BM aspirate is a highly invasive and painful procedure making it an undesirable source for long term use. Recently MSC have been isolated from clinical waste tissues such as umbilical cord that exert similar effects as BMMSC (Christodoulou *et al.*, 2013; Baksh *et al.*, 2007; Jin *et al.*, 2013; Roemeling-van Rhijn *et al.*, 2013). Therefore, we assessed whether umbilical cord-derived WJMSC or trabecular bone-derived TBMSC (taken from patients after hip replacement surgery) could induce the same suppressive effect on recruitment as BMMSC. We observed that MSC from clinical waste tissue could suppress neutrophil recruitment to the same extent as BMMSC.

The models used in this chapter mimic the action of MSC in co-culture with EC in different contexts. The microslide channel model, incorporating MSC into the EC monolayer does not occur physiologically but may arise after systemic infusion of MSC as a therapeutic intervention. Our data suggests that systemic infusion of early passage WJMSC or TBMSC in patients with chronic inflammation may suppress the influx of circulating leukocytes to the same extent as BMMSC. The filter model, where

MSC and EC were separated by a porous membrane mimics direct injection of MSC into the tissue for therapy. This indicates that WJMSC and TBMSC were able to suppress neutrophil recruitment when in reduced contact with the EC. However, close proximity was critical as separation of these MSC populations from EC reduced their suppressive effects. This suggests that the optimum method for administration of WJMSC or TBMSC would be directly into the target tissue ensuring that the cells are injected into a highly vascularised site. Therefore, WJMSC and TBMSC are both attractive alternative MSC sources that can be used as a treatment for chronic inflammation by inhibiting the aberrant recruitment of circulating leukocytes. Our results also suggest that treatment with co-culture conditioned medium may be just as effective as direct MSC infusion for treatment of chronic inflammation.

The filter model also mimics the interactions between EC and MSC *in vivo* as MSC are generally located in the perivascular niche in close proximity to EC (Méndez-Ferrer *et al.*, 2010). Therefore, we showed that as well as their reparative functions, tissue-resident MSC may also suppress the aberrant recruitment of neutrophils in response to cytokines in an attempt to dampen inflammation. Previous studies have shown that other tissue-resident stromal cells are also capable of inducing this effect (Kuravi *et al.*, 2014; McGettrick *et al.*, 2009). Thus, we conclude that MSC in this context are a model of tissue stroma which is an inherently suppressive microenvironment that acts to limit leukocyte infiltration during inflammation. It has previously been reported that stromal cells (i.e. fibroblasts) from chronically inflamed sites lose their capacity to suppress leukocyte recruitment in response to changes in their local microenvironment [reviewed by (McGettrick *et al.*, 2012)]. It would be interesting to test the effects of chronic inflammation on the capacity of MSC to regulate recruitment.

In conclusion, we have shown that MSC from three different sources are capable of suppressing neutrophil recruitment to inflamed endothelium in an IL-6 and TGF $\beta$ -dependent manner. The effect of MSC co-culture could be achieved by co-culture conditioned media and was dependent upon proximity between MSC and EC and their effects were diminished with passage. Based on these data we propose that MSC act as endogenous regulators of the response of EC to cytokines which may limit recruitment of the inflammatory infiltrate.

## **5. Effect of differentiation on the capacity of MSC to regulate leukocyte recruitment**

## 5.1. Introduction

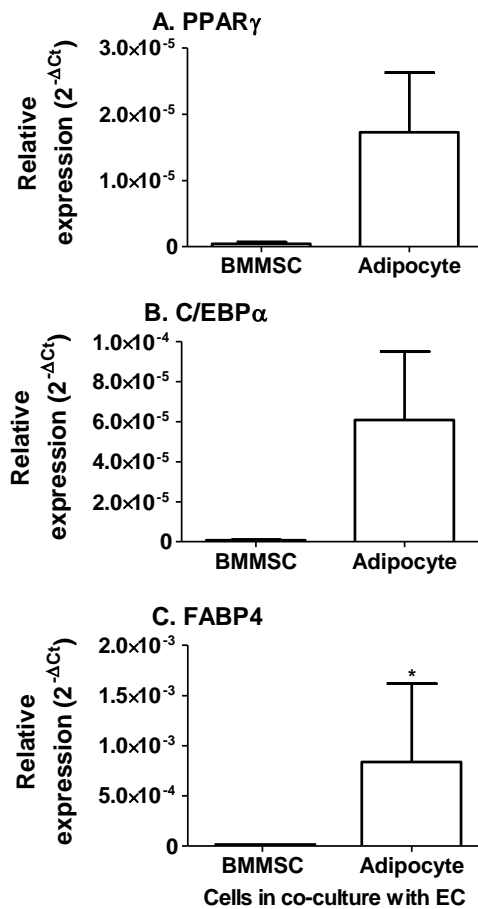
MSC differentiation *in vivo* is thought to be a normal physiological process in response to tissue damage. However, recent evidence suggest that MSC may aberrantly differentiate (namely into adipocytes and osteoblasts) in chronically inflamed tissue, potentially contributing to ectopic fat and bone commonly found at these sites (Uezumi *et al.*, 2010; Breitbach *et al.*, 2007). While the immune-protective functions of MSC have been extensively studied, the effect of differentiation on these responses have not been well characterised. We hypothesised that MSC lose their ability to suppress leukocyte recruitment after adipogenic differentiation which may contribute to chronic inflammation.

Using the filter-based adhesion assay, we examined whether BMMSC-derived adipocytes regulate the recruitment of leukocytes from flow and the mechanism involved in mediating their effects. We then used a 3D collagen gel model to assess whether adipocyte co-culture with EC influences neutrophil migration into tissue. 3D collagen gel assays performed with microvascular blood EC (BEC) and gene expression analysis of adhesion markers by EC in co-culture with MSC and adipocytes were performed by my project student under my supervision. Finally, we examined whether subcutaneous fat-derived pre-adipocytes (pre-AD; before and after *in vitro* adipogenic differentiation) and mature adipocytes (AD) share similar characteristics as BMMSC-derived adipocytes, in terms of their ability to modulate leukocyte recruitment from flow.

## 5.2. Results

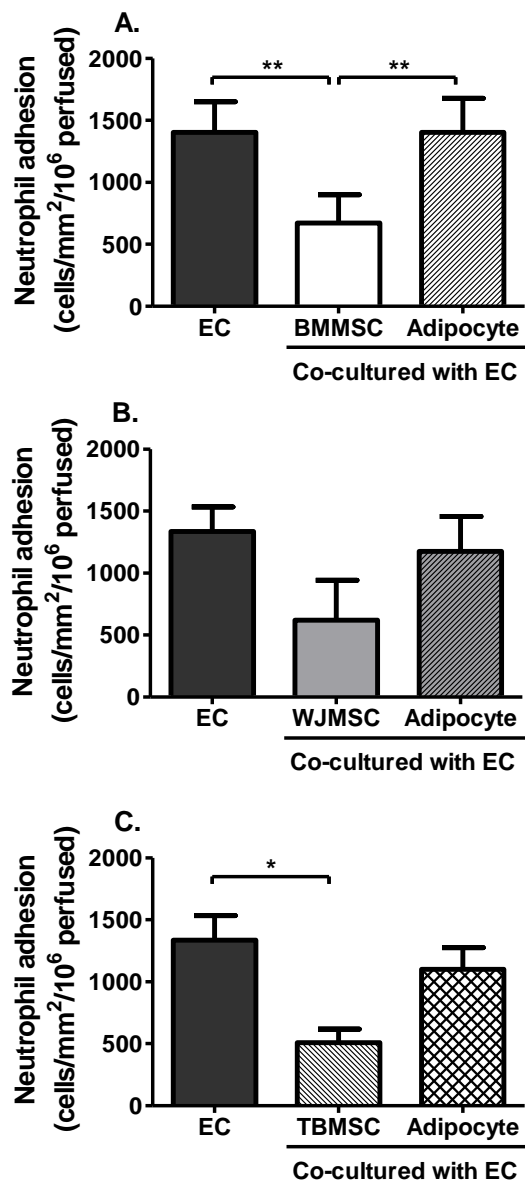
### 5.2.1. *Effects of MSC or MSC-derived adipocyte culture on leukocyte recruitment*

Initially, MSC were differentiated for 21 days using adipogenic induction media, we then examined expression of adipocyte markers, PPAR $\gamma$ , C/EBP $\alpha$  and FABP4 in BMMSC and BMMSC-derived adipocyte mono-cultures to confirm that they were upregulated upon differentiation. Expression of all 3 markers were increased upon differentiation compared to undifferentiated BMMSC mono-cultures, although only the increase in FABP4 expression was statistically significant (Figure 5-1). Adipocyte marker expression by WJMSC and TBMSC was not analysed as there were insufficient cells to perform these analyses. Then we tested the effect of adipogenic differentiation on the ability of MSC from different sources to suppress neutrophil adhesion. Adipogenic differentiation of BMMSC, WJMSC and TBMSC reduced their ability to suppress neutrophil adhesion to TNF $\alpha$ -stimulated EC compared to undifferentiated MSC, although the loss of suppression was only significant with BMMSC (Figure 5-2). Differentiation appeared to have no significant effect on the behaviour of adherent neutrophils compared to MSC co-culture (Figure 5-3). BMMSC exhibited a greater adipogenic differentiation potential than WJMSC (Section 3.4.) and were therefore used in all subsequent assays. Subsequently, we assessed the effect of BMMSC-derived adipocyte co-culture with EC on PBL recruitment after 24h TNF $\alpha$  and IFN $\gamma$ -stimulation. In contrast, to their effects on neutrophil recruitment, co-culture with BMMSC-derived adipocytes significantly suppressed PBL adhesion to the same extent as BMMSC (Figure 5-4A), but had no effect on their subsequent behaviour, when compared to EC mono-cultures (Figure 5-4B). Therefore, while adipogenic differentiation alters their capacity to regulate neutrophil recruitment, the effects of BMMSC on PBL recruitment were unaffected by differentiation.

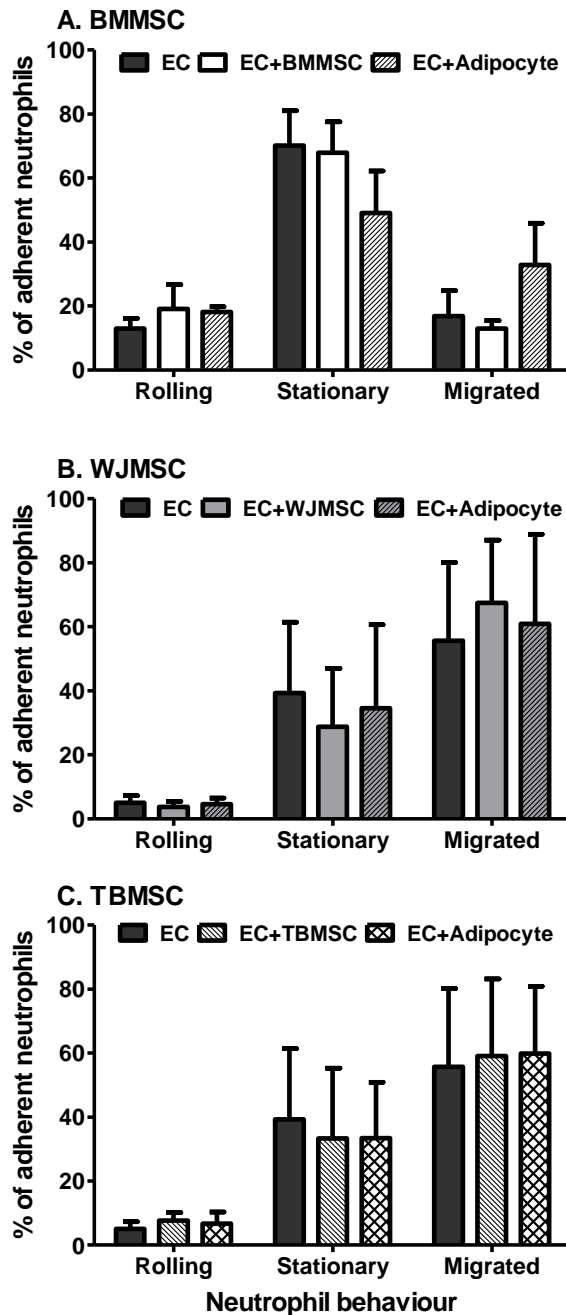


**Figure 5-1: Expression of adipocyte markers by BMMSC after adipogenic differentiation.** BMMSC were cultured in the presence or absence of adipogenic induction media for 21 days. Gene expression for the adipocyte markers (A) PPAR $\gamma$ , (B) C/EBP $\alpha$  and (C) FABP4 were examined by qPCR. Data are mean  $\pm$  SEM from n= 3 independent experiments. In A and B, paired t-test showed no significant effect of differentiation on gene expression. In C, paired t-test showed a significant change in gene expression upon differentiation;  $p < 0.05$ . 2 different BMMSC donors were used in this set of experiments.

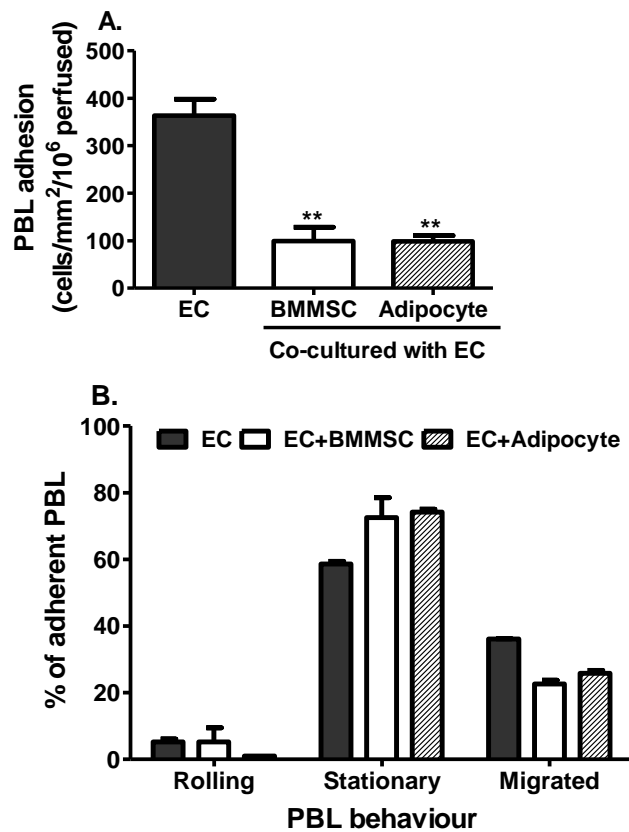




**Figure 5-2: Effect of adipogenic differentiation of MSC on neutrophil adhesion.** BMMSC, WJMSC and TBMSC were cultured in adipogenic induction media for 21 days, seeded onto Transwell filters and co-cultured with EC for 24h. Parallel MSC co-cultures were also set up as a control. Cultures were stimulated with 100U/ml TNF $\alpha$  for 4h and neutrophil adhesion to (A) BMMSC, (B) WJMSC and (C) TBMSC undifferentiated and differentiated co-cultures was examined. Data are mean  $\pm$  SEM from n= 3 independent experiments. In A and C, ANOVA showed a significant effect of culture conditions on neutrophil adhesion,  $p < 0.01$  and  $p < 0.05$  respectively. \* =  $p < 0.05$  and \*\* =  $p < 0.01$  by Bonferroni post-test. In B, ANOVA showed no significant effect of culture conditions on neutrophil adhesion. A different EC and neutrophil donor was used per experiment. 2 different BMMSC and 3 different WJMSC and TBMSC donors were used in this set of experiments.



**Figure 5-3: Effect of adipogenic differentiation of MSC on neutrophil behaviour.** BMMSC, WJMSC and TBMSC were cultured in adipogenic induction media for 21 days, seeded onto Transwell filters and co-cultured with EC for 24h. Parallel MSC co-cultures were also set up as a control. Cultures were stimulated with 100U/ml TNF $\alpha$  for 4h and the behaviour of adherent neutrophils was examined in (A) BMMSC, (B) WJMSC and (C) TBMSC undifferentiated and differentiated co-cultures. Neutrophil behaviour was expressed as the percentage of adherent neutrophils that were rolling, stationary or migrated. Data are mean  $\pm$  SEM from n= 3 independent experiments. ANOVA showed no significant effect of culture conditions on neutrophil behaviour. A different EC and neutrophil donor was used per experiment. 2 different BMMSC and 3 different WJMSC and TBMSC donors were used in this set of experiments.



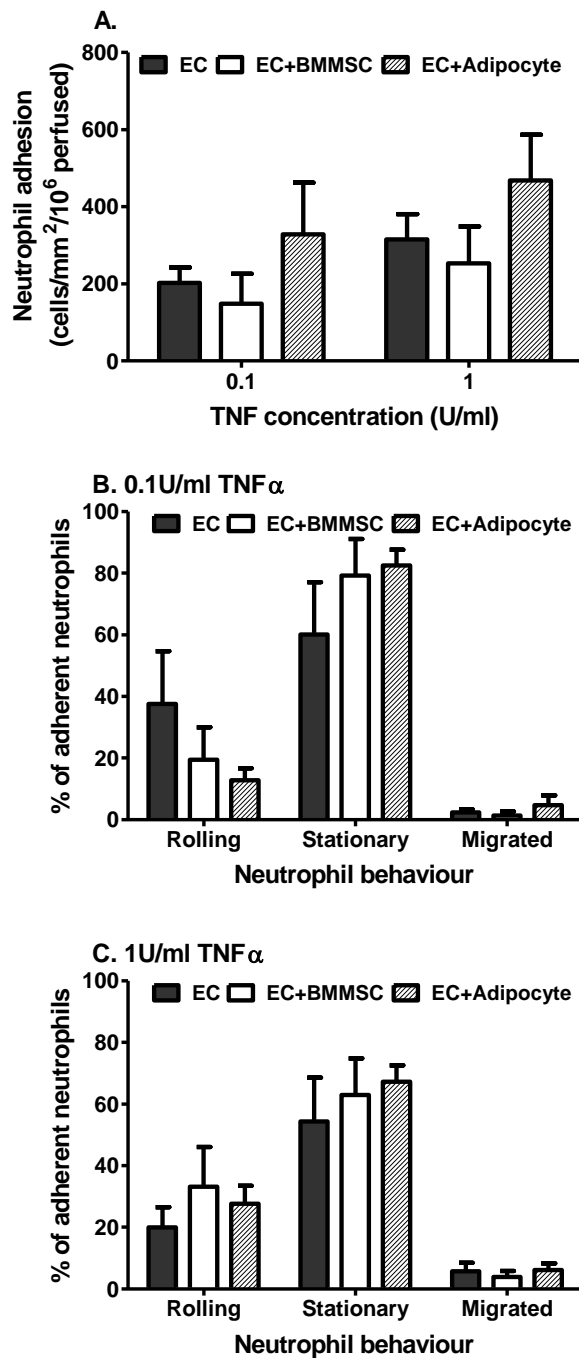
**Figure 5-4: Effect of adipogenic differentiation of MSC on PBL recruitment.** BMMSC were cultured in adipogenic induction media for 21 days, seeded onto Transwell filters and co-cultured with EC for 24h. Parallel MSC co-cultures were also set up as a control. Cultures were stimulated with 100U/ml TNF $\alpha$  and 10ng/ml IFN $\gamma$  for 24h. PBL (A) adhesion and (B) behaviour were analysed. PBL behaviour was expressed as the percentage of adherent neutrophils that were rolling, stationary or migrated. Data are mean  $\pm$  SEM from n= 3 independent experiments. In A, ANOVA showed a significant effect of culture conditions on PBL adhesion,  $p < 0.01$ . \*\* =  $p < 0.01$  by Dunnett post-test compared to EC mono-cultures. In B, ANOVA showed no significant effect of culture conditions on PBL behaviour. A different EC and PBL donor was used per experiment and 2 different BMMSC were used in this set of experiments.

### *5.2.2. Effect of adipogenic differentiation on neutrophil recruitment in response to low-grade inflammation*

While BMMSC-derived adipocytes lose their capacity to suppress neutrophil recruitment at high dose TNF $\alpha$  (100U/ml), they did not enhance neutrophil adhesion either. We hypothesised that at high dose TNF $\alpha$ , the maximum level of recruitment by EC may have been reached and so any pro-inflammatory effects would be masked. Therefore, BMMSC and differentiated adipocytes were co-cultured with EC and treated with lower doses of TNF $\alpha$  (0.1U/ml or 1U/ml). For both TNF $\alpha$  doses, low levels of neutrophil adhesion were observed on EC cultured alone or with BMMSC (Figure 5-5A). MSC co-culture did not significantly suppress neutrophil adhesion and co-culture with BMMSC-derived adipocytes tended to increase neutrophil adhesion in response to low dose TNF $\alpha$  when compared to EC mono-cultures, although this effect was not significant (Figure 5-5A). In all conditions, the majority of adherent neutrophils were rolling or stationary, with a low percentage of cells undergoing transmigration (Figure 5-5B and C). Co-culture had no significant effect on neutrophil behaviour (Figure 5-5B and C).

### *5.2.3. Effect of adipocyte co-culture on the expression of IL-6 signalling molecules*

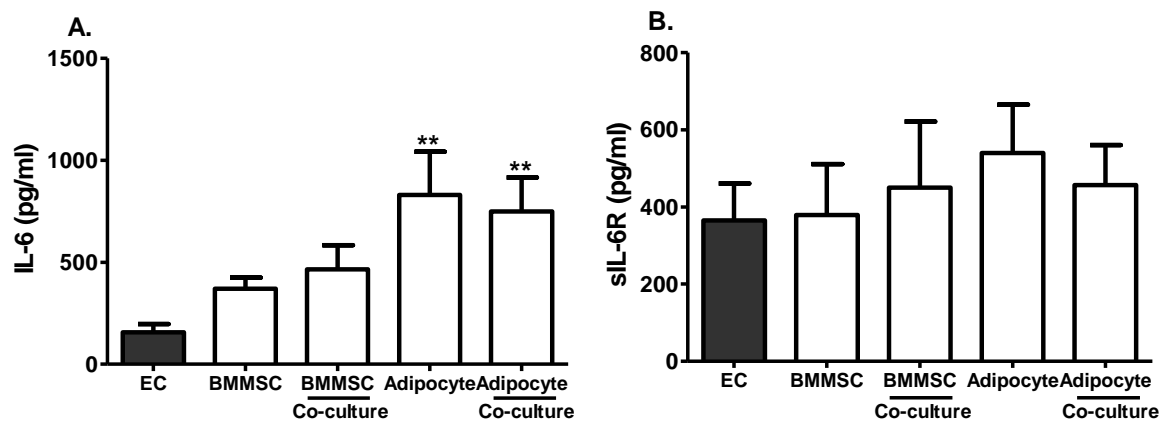
As we have previously shown that IL-6 was a key immunosuppressive mediator (Section 4.2.6.), we questioned whether secretion of IL-6 or its soluble receptors (sIL-6R) were reduced in MSC-derived adipocyte co-cultures and therefore could explain the loss of immunosuppression observed. Therefore, IL-6 and sIL-6R levels were measured in EC, BMMSC and BMMSC-derived adipocyte mono-culture and co-culture conditioned media after 24h.



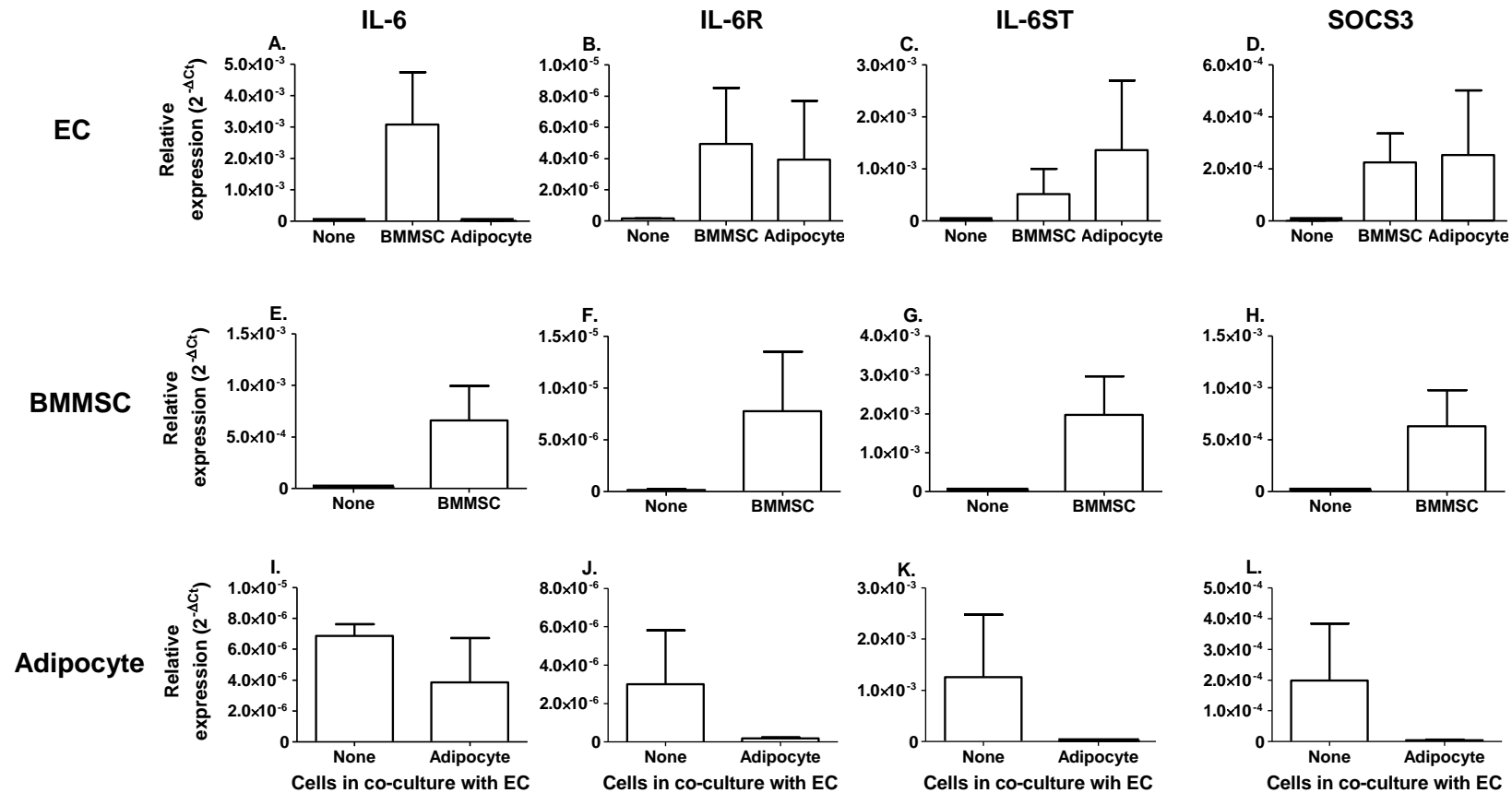
**Figure 5-5: Effect of adipogenic differentiation of MSC on neutrophil recruitment in response to low dose TNF $\alpha$ .** BMMSC were cultured in adipogenic induction media for 21 days, seeded onto Transwell filters and co-cultured with EC for 24h. Parallel MSC co-cultures were also set up as a control. Cultures were stimulated with 0.1U/ml or 1U/ml TNF $\alpha$  4h and neutrophil (A) adhesion and (B-C) behaviour were analysed. Neutrophil behaviour was expressed as the percentage of adherent neutrophils that were rolling, stationary or migrated. Data are mean  $\pm$  SEM from n= 3 independent experiments. ANOVA showed no significant effect of culture condition on neutrophil adhesion or behaviour. A different EC, BMMSC and neutrophil donor was used per experiment.

In contrast to BMMSC mono-cultures, conditioned media from adipocyte mono-cultures contained significantly higher levels of IL-6 than EC mono-cultures and co-culture with EC did not significantly alter its production (Figure 5-6A). sIL-6R levels on the other hand did not significantly change in any of the culture conditions (Figure 5-6B). Therefore, changes in IL-6 or sIL-6R secretion could not account for the loss of MSC-mediated suppression of neutrophil recruitment upon differentiation.

The gene expression of components of the IL-6 signalling pathway (IL-6, IL-6R, IL-6ST [signal transducer] and SOCS3 [an IL-6 inducible gene]) were also analysed to determine whether any changes in their expression in EC, MSC or adipocytes occurred during co-culture. IL-6 gene expression was elevated in EC from BMMSC co-cultures compared to EC cultured alone, or with adipocytes although the effect was not significant (Figure 5-7A). The receptors for IL-6 (IL-6R and IL-6ST) and SOCS3 were up-regulated in EC in co-culture with either BMMSC or adipocytes at the mRNA level, compared to EC cultured alone although the effect was not statistically significant (Figure 5-7B, C and D). Furthermore, IL-6, IL-6R, IL-6ST and SOCS3 gene expression tended to be slightly increased in BMMSC upon co-culture compared to BMMSC mono-cultures, although the increase was not statistically significant (Figure 5-7E-H). In contrast, these genes tended to be expressed at much lower levels in adipocytes during co-culture compared to mono-culture, however, the difference in gene expression was not statistically significant (Figure 5-7I-L). IL-6 gene expression remained high in adipocytes during co-culture (Figure 5-7I) suggesting that these cells secreted IL-6 during co-culture with EC, as EC did not upregulate IL-6 expression in co-culture.



**Figure 5-6: Levels of IL-6 and sIL-6R in EC, BMMSC and BMMSC-derived adipocyte mono-culture and co-culture supernatants.** (A) IL-6 and (B) sIL-6R levels were measured in supernatants from 24h mono-cultures or co-cultures of EC, BMMSC and BMMSC-derived adipocytes on Transwell filters. Data are mean  $\pm$  SEM from (A)  $n=9$  and (B)  $n=4$  independent experiments. In A, ANOVA showed a significant effect of culture conditions on IL-6 secretion,  $p<0.01$ . \*\* =  $p<0.01$  compared to EC mono-cultures by Dunnett post-test. In B, ANOVA showed no significant effect of culture conditions on sIL-6R levels in supernatants.



**Figure 5-7: Expression of IL-6 related genes by EC, MSC and adipocytes.** BMMSC were cultured in adipogenic induction media for 21 days, seeded onto Transwell filters and co-cultured with EC for 24h. Parallel MSC co-cultures were also set up as a control. RNA from each cell type was isolated and the expression of (A,E,I) IL-6, (B,F,J) IL-6R, (C,G,K) IL-6ST and (D,H,L) SOCS3 were analysed by qPCR in parallel EC, BMMSC and adipocytes mono-cultures and co-cultures. Data are mean  $\pm$  SEM from  $n=3$  independent experiments. Paired t-test showed no significant difference in gene expression for all conditions tested. A different EC donor was used per experiment and 2 different BMMSC donors were used in this set of experiments.

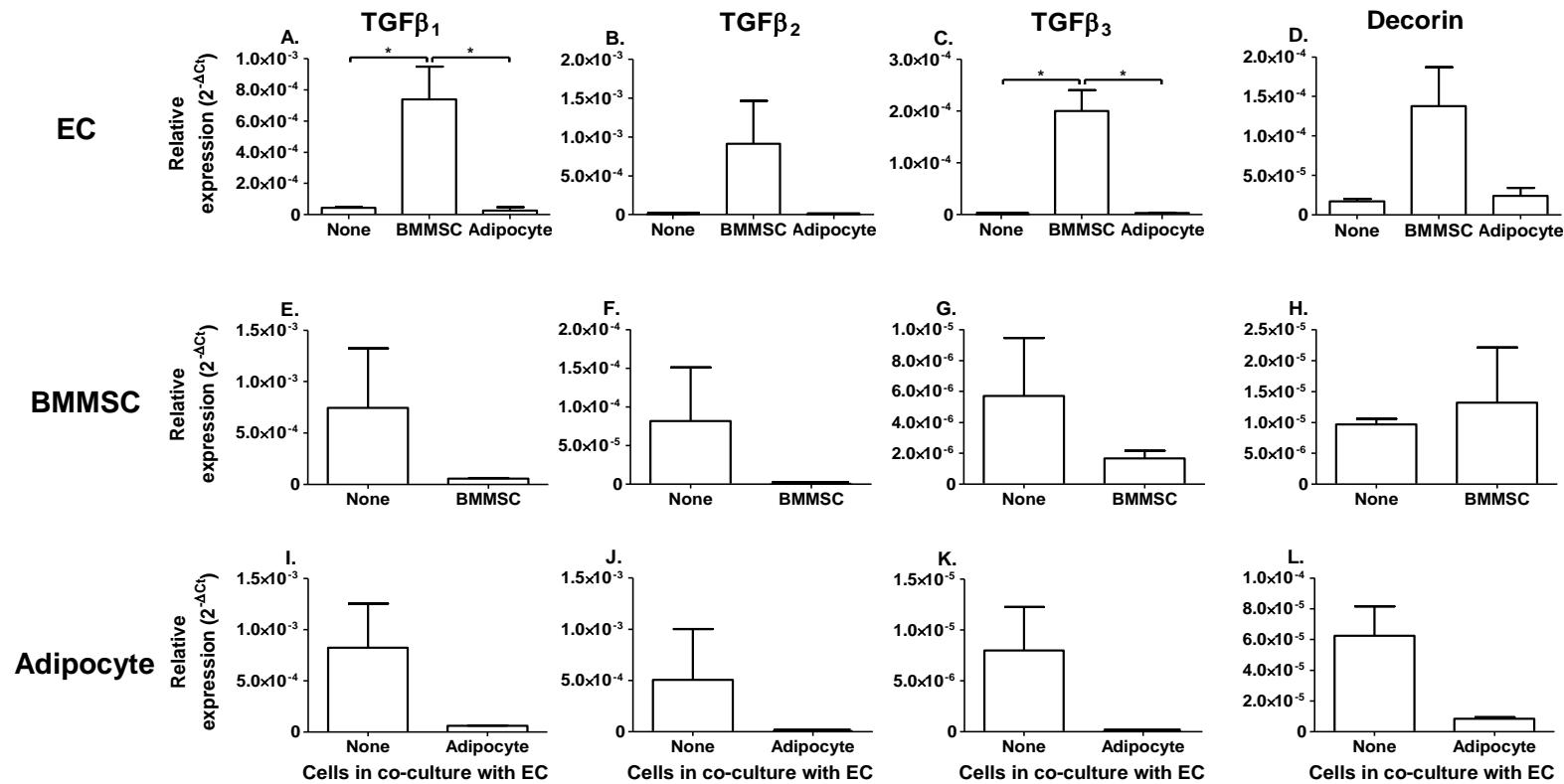


#### *5.2.4. Effect of adipocyte co-culture on the expression of TGF $\beta$ signalling molecules*

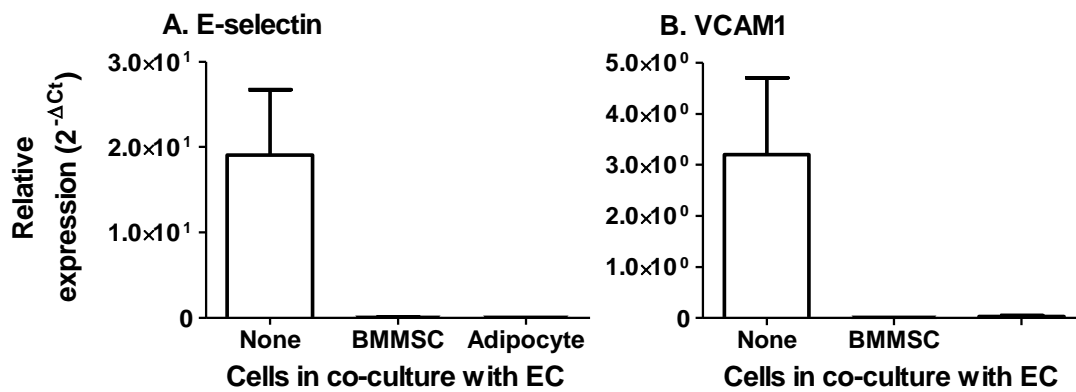
As we showed that the suppressive effects of BMMSC on neutrophil recruitment were mediated by the concerted action of IL-6 and TGF $\beta$  (Section 4.2.6.), we hypothesised that the loss of suppression upon differentiation may be due to a decrease in TGF $\beta$  expression in EC or adipocytes in co-culture. The expression of TGF $\beta_1$ , TGF $\beta_2$  and TGF $\beta_3$  and decorin (a TGF $\beta_1$  binding protein) was assessed in EC, BMMSC and adipocyte mono-cultures and co-cultures. The expression of all 3 TGF $\beta$  isoforms and decorin were upregulated in EC co-cultured with BMMSC but not adipocytes, however, only the increase in TGF $\beta_1$  and TGF $\beta_3$  expression was significant (Figure 5-8A-D). Expression of all TGF $\beta$  isoforms were highest in BMMSC and adipocyte mono-cultures and tended to be reduced by co-culture, although the change in expression was not statistically significant (Figure 5-8E-G and I-K). Decorin was expressed at similar levels by BMMSC mono-cultures and co-cultures (Figure 5-8H). In contrast, expression of decorin by adipocytes tended to be downregulated during co-culture although the effects were not significant (Figure 5-8L). This suggests that during adipocyte co-culture, while IL-6 secretion is still upregulated, TGF $\beta$  expression is suppressed and may not be present to mediate suppression of neutrophil recruitment along with IL-6.

#### *5.2.5. Effect of adipocyte co-culture on expression of adhesion molecules by EC*

We also analysed E-selectin and VCAM1 gene expression by TNF $\alpha$ -stimulated EC in co-culture, to assess whether MSC or MSC-derived adipocyte co-culture altered the expression of adhesion markers by EC. However, expression of E-selectin and VCAM1 was highest in EC mono-cultures and tended to be reduced in BMMSC and adipocyte co-culture, although the decrease was not statistically significant (Figure 5-9).



**Figure 5-8: Gene expression of TGFβ and decorin by EC, MSC and adipocytes.** BMMSC were cultured in adipogenic induction media for 21 days, seeded onto Transwell filters and co-cultured with EC for 24h. Parallel MSC co-cultures were also set up as a control. RNA from each cell type was isolated and the expression of (A,E,I) TGFβ<sub>1</sub>, (B,F,J) TGFβ<sub>2</sub>, (C,G,K) TGFβ<sub>3</sub> and (D,H,L) Decorin were analysed by qPCR. In parallel EC, BMMSC and adipocytes mono-cultures and co-cultures were also set up. Data are mean ± SEM from n= 3 independent experiments. In A and C, ANOVA showed a significant effect culture conditions on gene expression by EC, p<0.05. \* = <0.05 compared to BMMSC co-culture by Bonferroni post-test. In E-L, paired t-test showed no significant difference in gene expression for all conditions tested. A different EC donor was used per experiment and 2 different BMMSC donors were used in this set of experiments.



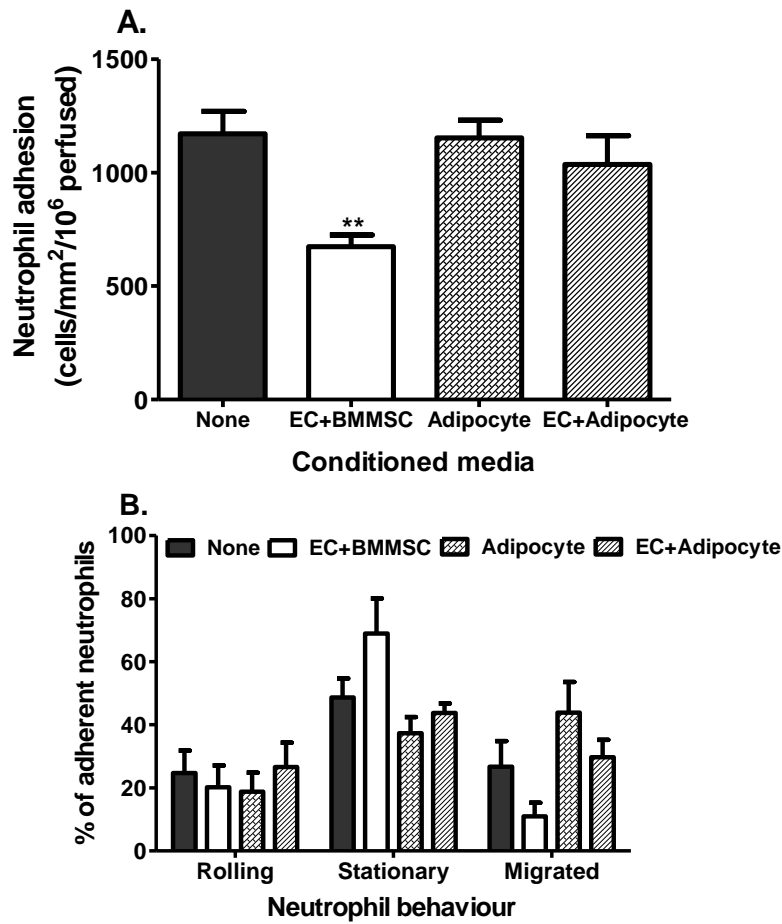
**Figure 5-9: Gene expression of adhesion markers by EC mono-cultures and co-cultures after stimulation with TNF $\alpha$ .** BMMSC were cultured in adipogenic induction media for 21 days, seeded onto Transwell filters and co-cultured with EC for 24h. Parallel MSC co-cultures were also set up as a control. All cultures were stimulated with TNF $\alpha$  for 2h. RNA from EC was isolated and the expression of (A) E-selectin and (B) VCAM1 were analysed by qPCR. Data are mean  $\pm$  SEM from n= 3 independent experiments. ANOVA showed no significant difference in gene expression for all culture conditions tested. A different EC donor was used per experiment and 2 different BMMSC donors were used in this set of experiments.

This suggests that the loss of suppression with adipocytes was not due to an increase in adhesion molecule expression on EC.

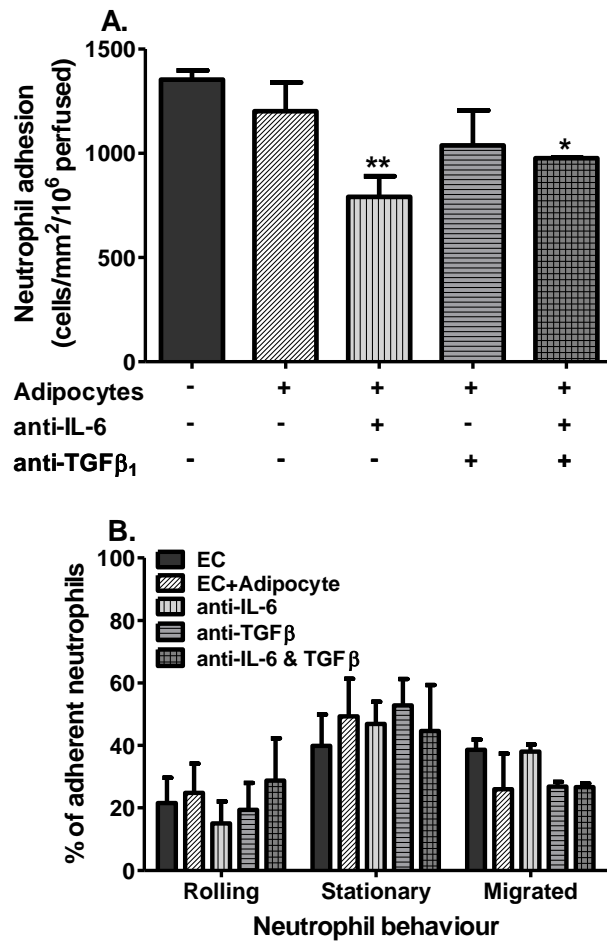
#### *5.2.6. Roles of soluble factors in the loss of suppression by MSC-derived adipocytes*

As IL-6 secretion was high in adipocyte co-culture, we wanted to assess whether adipocyte co-culture conditioned media suppressed recruitment to the same extent as BMMSC co-culture. Unlike BMMSC co-culture conditioned media which significantly suppressed neutrophil adhesion, treatment of EC with conditioned media from adipocyte mono-cultures or co-cultures had no statistically significant effect on neutrophil adhesion compared to EC cultured alone (Figure 5-10A). Neutrophil behaviour was also not significantly altered by treatment with all conditioned media (Figure 5-10B). Therefore, adipocyte co-culture conditioned media, despite containing high levels of IL-6 was unable to suppress neutrophil recruitment.

Next we examined the effect of neutralising IL-6 and TGF $\beta$  on neutrophil recruitment to BMMSC-derived adipocytes co-cultures to assess whether these factors play a role in the loss of suppression observed. Neutralisation of IL-6 alone or in combination with TGF $\beta$  in adipocyte co-cultures significantly suppressed neutrophil adhesion compared to EC mono-cultures (Figure 5-11A). In contrast, TGF $\beta$  neutralisation had no significant effect on neutrophil adhesion, which was to be expected given that its expression was reduced during adipocyte co-culture (Figure 5-8). Antibody treatment had no statistically significant effect on the behaviour of adherent neutrophils (Figure 5-11B). Therefore, loss of suppression by MSC upon differentiation was mediated by IL-6 but not TGF $\beta$ .



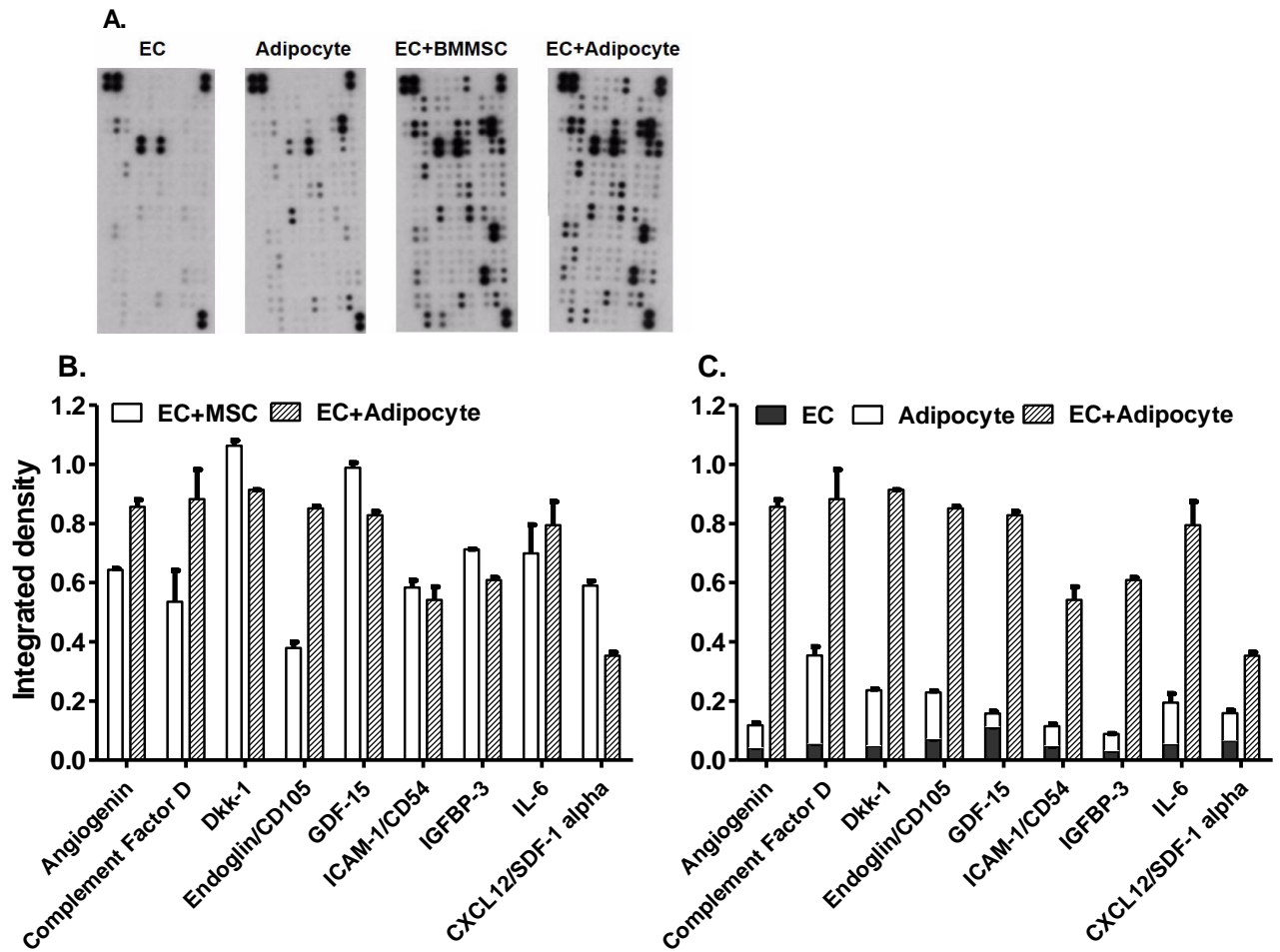
**Figure 5-10: Effect of adipocyte conditioned media on neutrophil recruitment.** Conditioned media were harvested from BMMSC and adipocytes cultured alone or with EC for 24h. EC mono-cultures were treated with conditioned media for 24h before stimulation with 100U/ml TNF $\alpha$  for 4h in the same culture media. Neutrophil (A) adhesion and (B) behaviour were analysed. Neutrophil behaviour was expressed as the percentage of adherent neutrophils that were rolling, stationary or migrated. Data are mean  $\pm$  SEM from n= 5 experiments. In A, ANOVA showed a significant effect of culture conditions on neutrophil adhesion,  $p < 0.01$ . \*\* =  $p < 0.01$  by Dunnett post-test compared to EC mono-cultures. In B, ANOVA showed no significant effect of culture conditions on neutrophil behaviour. A different EC and neutrophil donor was used per experiment and mono-culture and co-culture conditioned media from 3 different BMMSC donors were used in this set of experiments.



**Figure 5-11: Effect of neutralisation of IL-6 and TGFβ in adipocyte co-culture on neutrophil recruitment.** Adipocyte co-cultures were treated with neutralising antibodies against IL-6 or TGFβ alone or in combination for the duration of the co-culture and 4h cytokine treatment. Neutrophil (A) adhesion and (B) behaviour were assessed. Neutrophil behaviour was expressed as the percentage of adherent neutrophils that were rolling, stationary or migrated. Data are mean ± SEM from n= 3 independent experiments. In A, ANOVA showed a significant effect of culture conditions on neutrophil adhesion,  $p < 0.01$ . \* =  $p < 0.05$  and \*\* =  $p < 0.01$  by Dunnett post-test compared to EC mono-cultures. In B, ANOVA showed no significant effect of culture condition on neutrophil behaviour. A different EC, BMMSC and neutrophil donor was used per experiment.

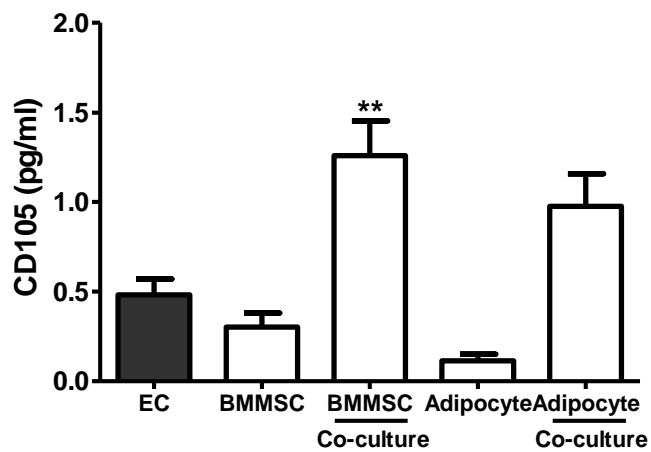
Subsequently, we used a cytokine expression array to detect the presence of 102 cytokines in 4 different conditioned media from one experiment (EC mono-culture, BMMSC co-culture and adipocyte mono-cultures and co-cultures). Our aim was to use this unbiased screen to identify differences in cytokine secretion between MSC and adipocyte co-cultures that could be responsible for the loss of immunosuppression observed (Figure 5-12A). Of the screened cytokines, Angiogenin, Complement factor D and CD105 were higher in adipocyte co-culture than BMMSC co-culture conditioned media (Figure 5-12B). In addition, adipocyte co-culture was found to increase secretion of several other cytokines, including Angiogenin, Complement factor D and CD105, compared to EC and adipocyte mono-cultures (Figure 5-12C).

As CD105 is an auxiliary TGF $\beta$  co-receptor, we hypothesised that increased shedding of CD105 in adipocyte co-cultures compared to MSC co-cultures reduced TGF $\beta$  signalling in co-culture, inhibiting the suppression of neutrophil recruitment. To validate the results of the array, we measured CD105 levels in EC, BMMSC and adipocyte mono-cultures and co-cultures by ELISA. BMMSC and adipocyte co-cultures had higher levels of CD105 compared to all mono-culture conditions, although it was only significant with BMMSC co-culture (Figure 5-13). Furthermore, the concentration of CD105 detected in all samples was very low (Figure 5-13). In contrast to the cytokine array data, we observed no statistically significant difference in the concentration of CD105 released during BMMSC or adipocyte co-culture with EC (Figure 5-13). Therefore, changes in CD105 do not account for the differences in immunosuppression observed between the two co-cultures.



**Figure 5-12: Comparison of cytokine secretion in EC, BMMSC and BMMSC-derived adipocyte mono-culture and co-culture supernatants.** 24h EC mono-culture, BMMSC co-culture, adipocyte mono-culture and co-culture conditioned media from one experiment were added to (A) membrane antibody arrays. The average pixel density for each cytokine was analysed. The integrated density of each analyte in (B) BMMSC vs. adipocyte co-cultures and (C) EC and adipocyte mono-cultures vs. adipocyte co-cultures were compared. In B and C, data are mean  $\pm$  SEM from duplicate analytes in  $n = 1$  experiment.

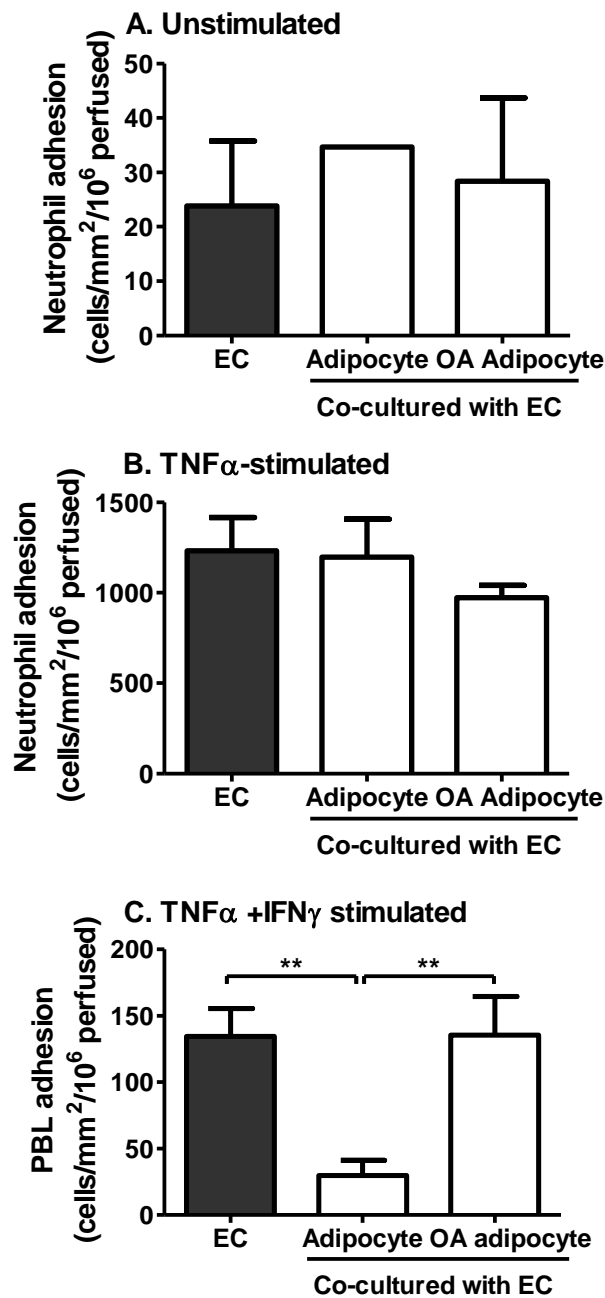




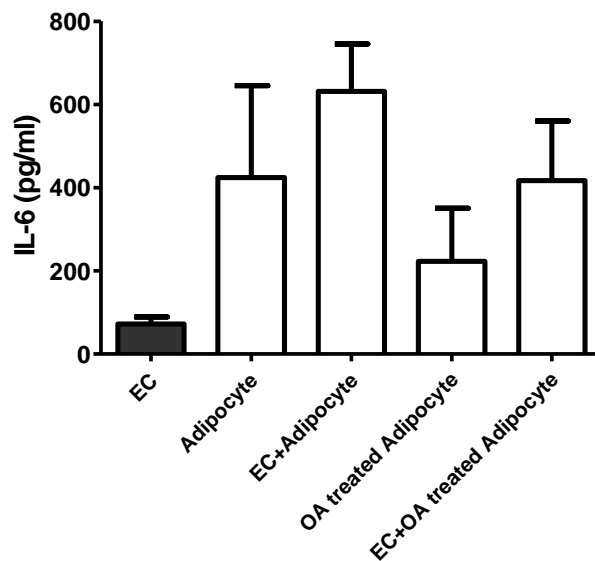
**Figure 5-13: Levels of CD105 in BMMSC and adipocyte mono-culture and co-culture supernatants.** CD105 was measured in supernatants from 24h mono-cultures or co-cultures of EC, BMMSC and BMMSC-derived on Transwell filters. Data are mean  $\pm$  SEM from n= 6 independent experiments. ANOVA showed a significant effect of culture conditions on CD105 levels,  $p < 0.01$  \*\* =  $p < 0.01$  compared to EC mono-cultures by Dunnett post-test.

#### *5.2.7. Effect of fatty acid treatment of MSC-derived adipocyte on leukocyte adhesion*

Next, we wanted to determine whether treatment of BMMSC-derived adipocytes with fatty acids (present in metabolic disorders) enhanced leukocyte adhesion by shifting the adipocytes into a pro-inflammatory state. BMMSC were differentiated on filters for 21 days and then treated with oleic acid (OA) for 24h prior to co-culture with EC. Neutrophil and PBL adhesion was examined. In these assays fluorescently labelled leukocytes were perfused over the endothelium because the lipid droplets within the differentiated MSC-derived adipocytes obscured the image on the filter making it difficult to visualise the adherent leukocytes. Consequently, leukocyte behaviour was not analysed as it was too difficult to distinguish stationary and transmigrated leukocytes under fluorescence. OA treatment had no significant effect on neutrophil adhesion to untreated or TNF $\alpha$  treated adipocyte co-cultures when compared to matched EC controls (Figure 5-14A and B). Co-culture with BMMSC-derived adipocytes significantly suppressed PBL adhesion (Figure 5-14C). In contrast, treating adipocytes with OA reversed their ability to suppress PBL adhesion in response to cytokines (Figure 5-14C). The increase in PBL adhesion was statistically significant compared to untreated BMMSC-derived co-cultures. This suggests that OA treatment may cause adipocytes to become pro-inflammatory by abrogating their ability to suppress PBL recruitment. OA treatment tended to reduce the concentration of IL-6 measured in adipocyte mono-culture and co-culture conditioned media, but the effect was not significant (Figure 5-15).



**Figure 5-14: Effect of OA treatment of adipocytes on neutrophil and PBL adhesion.** BMMSC were adipogenically differentiated for 21 days and seeded onto filters. Adipocytes were treated with or without OA for 24h prior to seeding the EC. Neutrophil adhesion to (A) unstimulated and (B) 100U/ml TNF $\alpha$ -stimulated EC were analysed. (C) PBL adhesion to EC mono-cultures and adipocyte co-cultures treated with 100U/ml TNF $\alpha$  and 10ng/ml IFN $\gamma$  was also analysed. Data are mean  $\pm$  SEM from n= 3 independent experiments. In A and B, ANOVA showed no significant effect of culture conditions on neutrophil adhesion. In C, ANOVA showed a significant effect of culture conditions on PBL adhesion, p<0.01. \*\* = p<0.01 by Bonferroni post-test compared to EC mono-cultures. A different EC and leukocyte donor was used per experiment and 2 different BMMSC donors were used in this set of experiments.



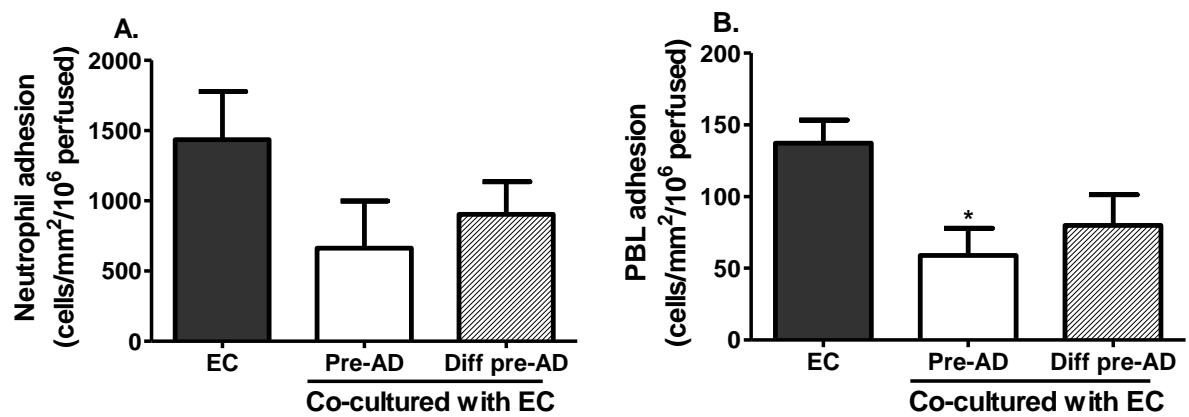
**Figure 5-15: Levels of IL-6 in EC and adipocyte mono-culture and co-culture supernatants after OA treatment.** BMMSC were adipogenically differentiated for 21 days and seeded onto filters. Adipocytes were treated with or without OA for 24h prior to seeding the EC. Conditioned media from 24h mono-cultures and co-cultures were harvested and IL-6 was measured. Data are mean  $\pm$  SEM from  $n=3$  independent experiments. ANOVA showed no significant effect of culture conditions on IL-6 secretion.

#### *5.2.8. Effects of human adipocytes on leukocyte recruitment*

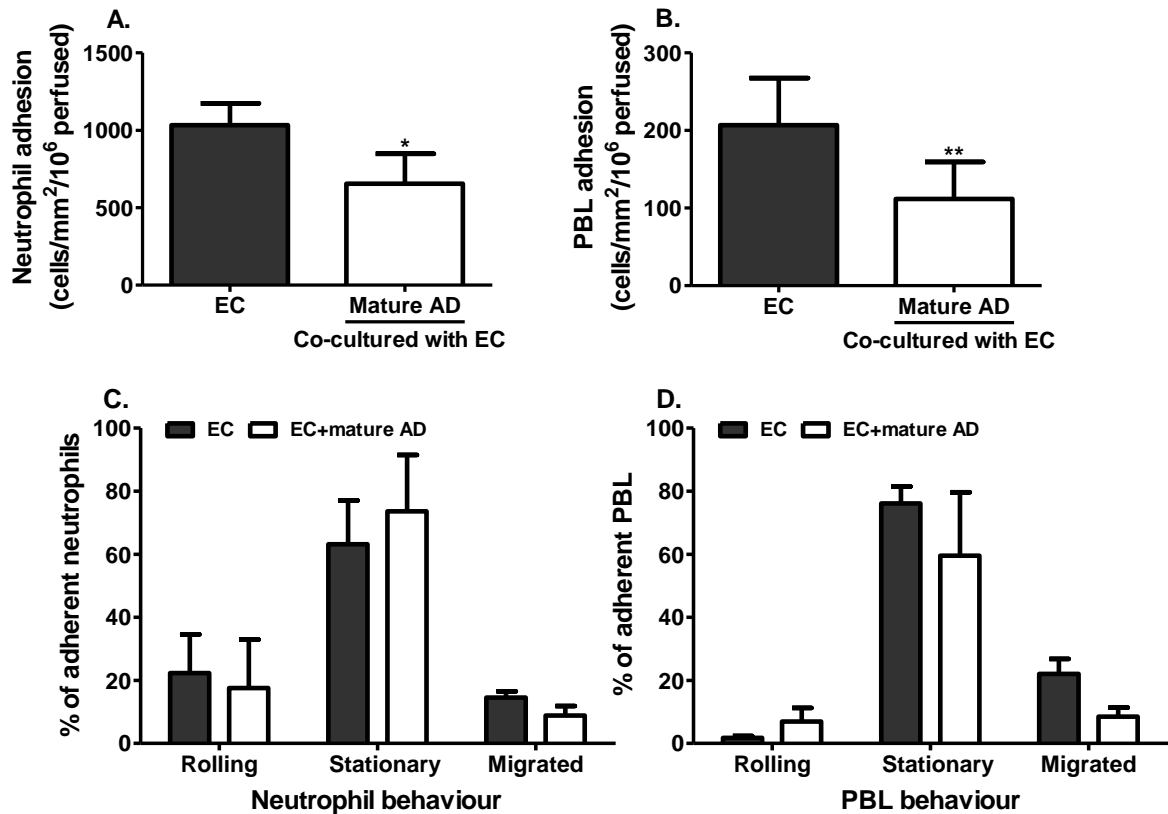
We then assessed the effects of adult human pre-AD and mature AD on neutrophil and PBL adhesion to determine whether they exert the same effects as BMMSC-derived adipocytes. Co-culture with pre-AD tended to suppress neutrophil adhesion compared to EC mono-cultures, although this was not significant (Figure 5-16A). Pre-AD significantly suppressed PBL adhesion compared to EC cultured alone (Figure 5-16B). Unlike BMMSC, pre-AD tended to suppress neutrophil and PBL adhesion after adipogenic differentiation, although again the effect was not significant (Figure 5-16). Co-culture with mature AD also significantly suppressed neutrophil and PBL adhesion compared to EC mono-cultures (Figure 5-17A and B) but had no statistically significant effect on the behaviour of adherent neutrophils (Figure 5-17C and D). Therefore, BMMSC-derived adipocytes do not exert the same effect on recruitment as actual human pre-AD and mature AD. However much like BMMSC-derived adipocytes, pre-AD and mature AD mono-cultures secreted significantly higher levels of IL-6 than EC mono-cultures and co-culture did not alter its secretion (Figure 5-18).

#### *5.2.9. Effect of BMMSC and BMMSC-derived adipocyte co-culture on the behaviour of migrated neutrophils*

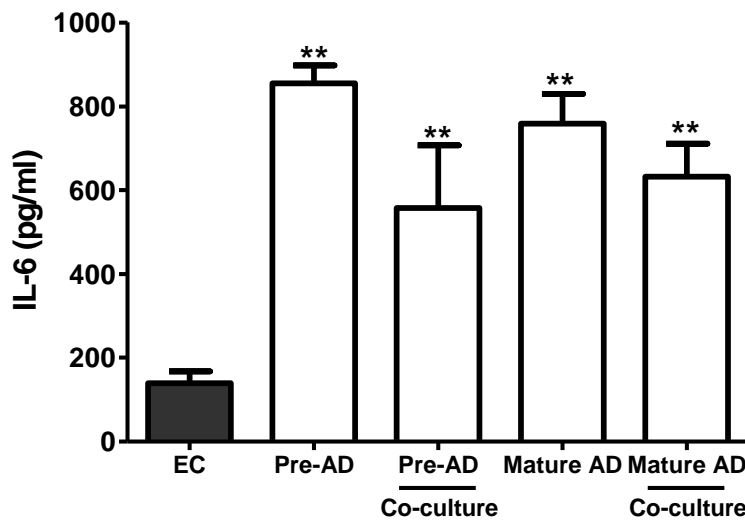
In a small series of experiments we also analysed the effects of BMMSC-derived adipocytes on the onward migration of neutrophils through a 3D collagen gel matrix as we wondered whether they could alter neutrophil migration. In response to TNF $\alpha$ -stimulation, co-culture with BMMSC suppressed neutrophil adhesion at 20min and 120min, where at the latter time point adhesion was significantly reduced compared to EC controls (Figure 5-19A and B).



**Figure 5-16: Effect of undifferentiated and differentiated pre-AD on neutrophil and PBL adhesion.** Pre-AD isolated from subcutaneous fat was seeded onto Transwell filters and cultured in adipogenic induction media for 21 days. EC were seeded onto the filters and co-cultured for 24h. Parallel undifferentiated pre-AD co-cultures were also set up. (A) Neutrophil and (B) PBL adhesion to EC treated with 100U/ml TNF $\alpha$  or 100U/ml TNF $\alpha$  and 10ng/ml IFN $\gamma$  respectively was analysed. Data are mean  $\pm$  SEM from n= 3 independent experiments. In A, ANOVA showed no significant effect of culture conditions on neutrophil adhesion. In B, ANOVA showed a significant effect of culture conditions on PBL adhesion, p<0.05. \* = p<0.05 by Bonferroni post-test compared to EC mono-cultures. A different EC, leukocyte and pre-AD donor was used per experiment.

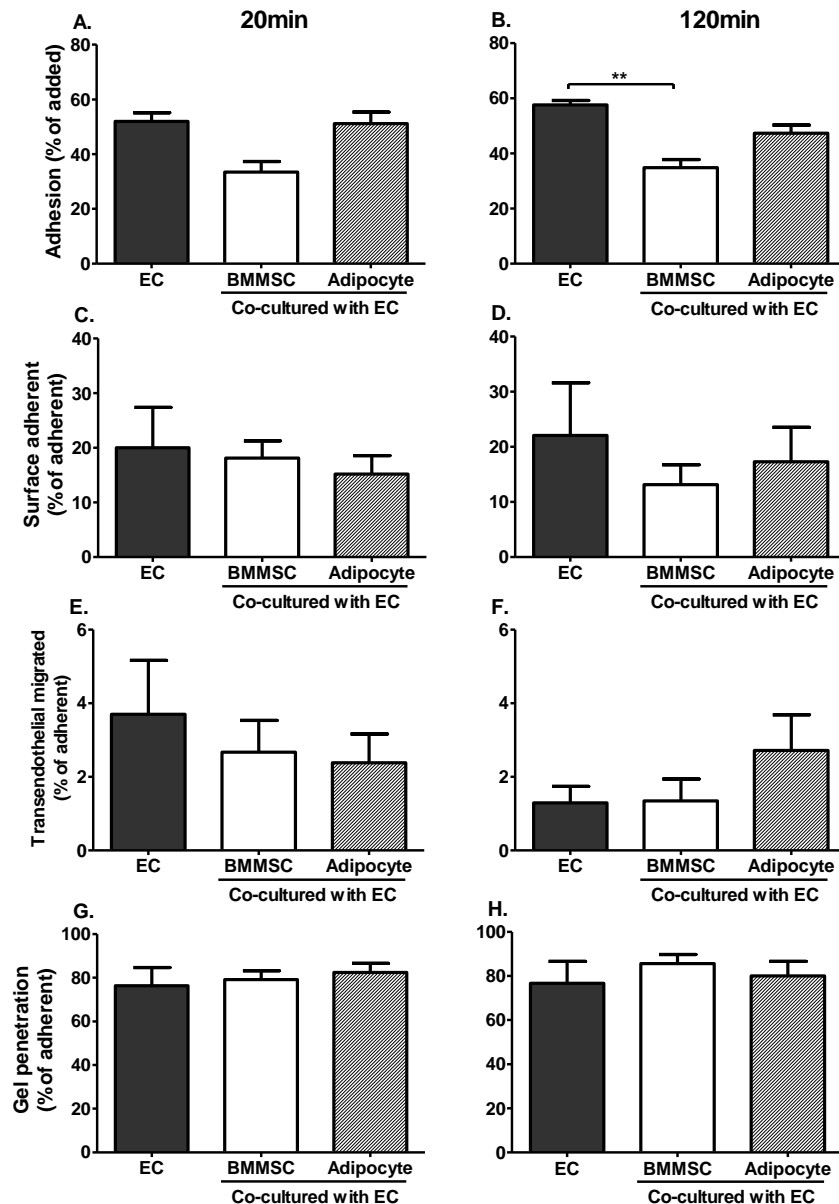


**Figure 5-17: Effect of mature AD on neutrophil and PBL recruitment.** Mature AD isolated from subcutaneous fat were added to a 6-well plate. EC were seeded above them onto a Transwell filter and co-cultured for 24h. (A,C) Neutrophil and (B,D) PBL adhesion and behaviour to EC treated with 100U/ml TNF $\alpha$  or 100U/ml TNF $\alpha$  and 10ng/ml IFN $\gamma$  respectively was analysed. Data are mean  $\pm$  SEM from n= 4 independent experiments. In A and B, paired t-test showed a significant effect of mature AD co-culture on neutrophil and PBL adhesion respectively. \* = p<0.05 and \*\* = p<0.01. In C and D, paired t-test did not show a significant effect of mature AD co-culture on neutrophil and PBL behaviour respectively. A different EC, leukocyte and mature AD donor was used per experiment.



**Figure 5-18: Levels of IL-6 in EC, pre-AD and mature AD mono-culture and co-culture supernatants.** IL-6 was measured in supernatants from mono-cultures or co-cultures of EC, pre-AD and mature AD cultured for 24h on Transwell filters. Data are mean  $\pm$  SEM from  $n=5$  independent experiments. ANOVA showed a significant effect of culture conditions on IL-6 secretion,  $p<0.01$ . \*\* =  $p<0.01$  compared to EC mono-cultures by Dunnett post-test.



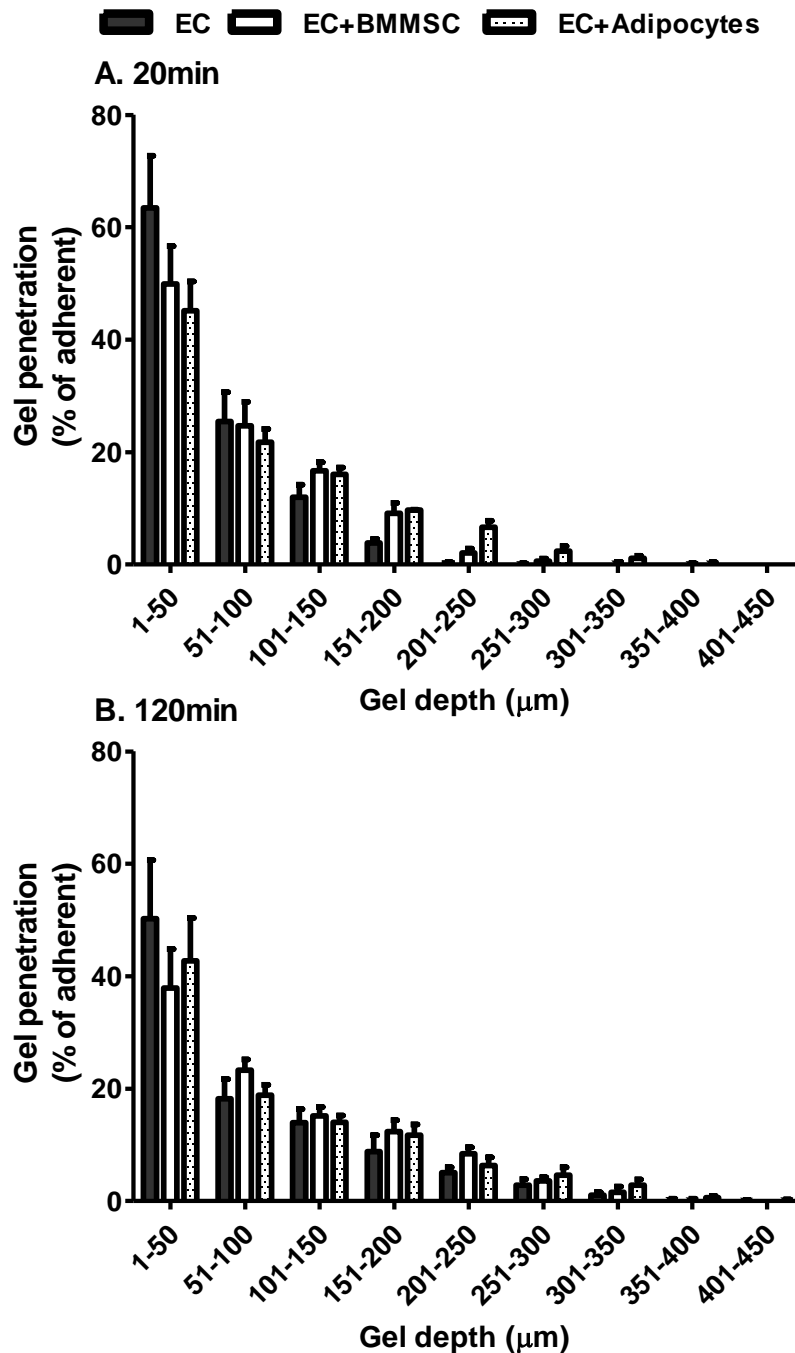


**Figure 5-19: Effect of adipocyte differentiation on neutrophil adhesion and migration into 3D collagen gels in response to  $\text{TNF}\alpha$ .** BMMSC or adipocytes were incorporated into collagen gels for 24h. EC were then seeded onto the gels for a further 24hr and were stimulated with 100U/ml  $\text{TNF}\alpha$  for 4hr. Neutrophils ( $5 \times 10^5$ ) were added to gels for 20min, non-adherent cells were washed off. Neutrophil adhesion and migration was assessed after 20min and 120min. (A,B) The percentage of neutrophils that were added to the gels that adhered after 20min and 120min respectively. The percentage of adherent neutrophils that were (C,D) surface adherent, (E,F) transendothelial migrated and (G,H) penetrated into the gel were assessed. Data are mean  $\pm$  SEM from  $n = 3$  experiments. In B, ANOVA showed a significant effect on culture conditions,  $p < 0.01$ , on the percentage of adherent neutrophils.  $**p < 0.01$  by Bonferroni post-test. In C-H, ANOVA showed no significant effect of culture conditions on neutrophil migration through gels. A different EC, neutrophil and BMMSC donor was used per experiment.

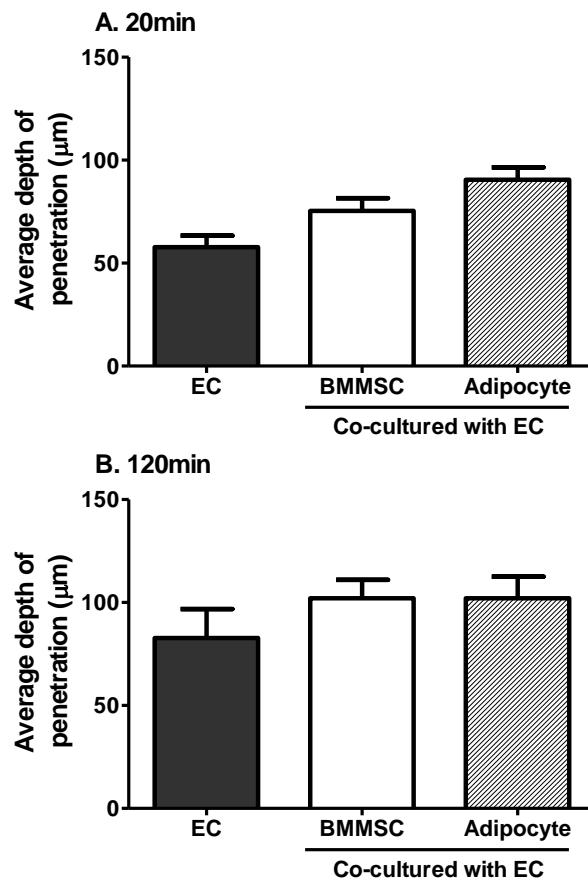
In agreement with the filter flow assay, adipogenic differentiation of BMMSC abrogated their suppressive effects on neutrophil adhesion at both time points (Figure 5-19A and B). Of those adherent cells, ~20% were surface bound and ~4% had migrated just under the endothelium, with the majority (80%) of cells having migrated into the gel at 20min and 120min (Figure 5-19C-H).

The behaviour of adherent neutrophils was not significantly affected by co-culture at both time points (Figure 5-19C-H). Most neutrophils that penetrated the gel tended to stay close to the surface after 20min with the average depth of penetration being ~55µm (Figure 5-20A and Figure 5-21A). After 120min, the migrated neutrophils migrated slightly deeper into the gel and the average depth of penetration was increased to ~90µm (Figure 5-20B and 5-21B). Co-culture with BMMSC and BMMSC-derived adipocytes had no significant effect on the depth of neutrophil penetration (Figure 5-20 and 5-21). Interestingly, similar effects on neutrophil adhesion and subsequent migration into gels were observed when MSC-derived adipocyte (Appendix 1-1 to 1-3) and MSC-derived osteoblasts (Appendix 1-3 to 1-6) co-cultures were formed with BEC. Therefore, MSC lost the ability to suppress neutrophil adhesion upon differentiation but had no effect on the onward migration of neutrophils into gels.

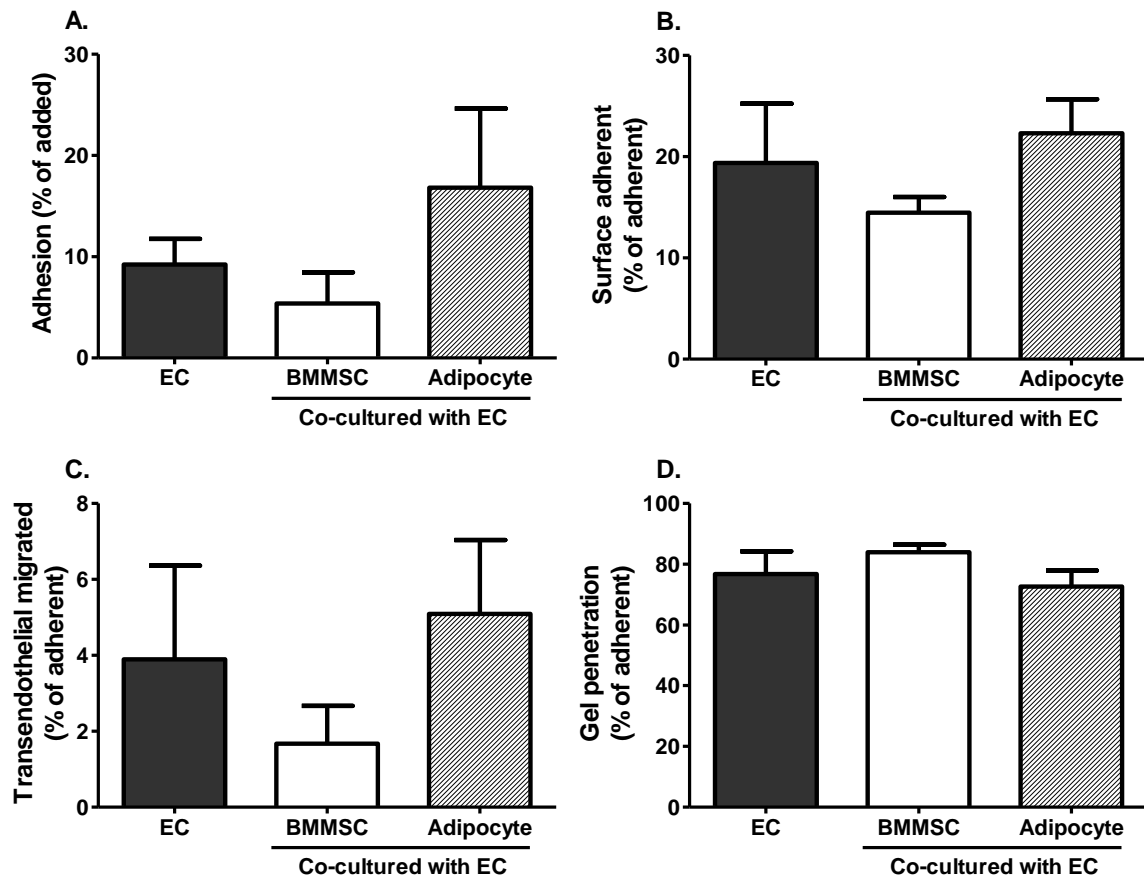
To assess whether MSC-derived adipocytes were pro-inflammatory, neutrophil adhesion and onward migration into gels in the absence of cytokines was analysed. Adipocytes tended to support binding of a higher percentage of neutrophils compared to EC cultured alone or with BMMSC, although the numbers were low and there was no significant difference between conditions (Figure 5-22A).



**Figure 5-20: Effect of adipocytes co-culture on the position of migrated neutrophils within 3D collagen gels in response to TNF $\alpha$ .** BMMSC or adipocytes were incorporated into collagen gels for 24h. EC were then seeded onto the gels for a further 24hr and were stimulated with 100U/ml TNF $\alpha$  for 4hr. Neutrophils ( $5 \times 10^5$ ) were added to gels for 20min and then non-adherent neutrophils were washed off. The position of neutrophils that penetrated into the gel were analysed by taking z-stack images at 2 $\mu$ m intervals through the gel after (A) 20min and (B) 120min. Data are mean  $\pm$  SEM from  $n = 3$  experiments. ANOVA showed no significant effect of culture conditions on neutrophil migration through gels. A different EC, neutrophil and BMMSC donor was used per experiment.

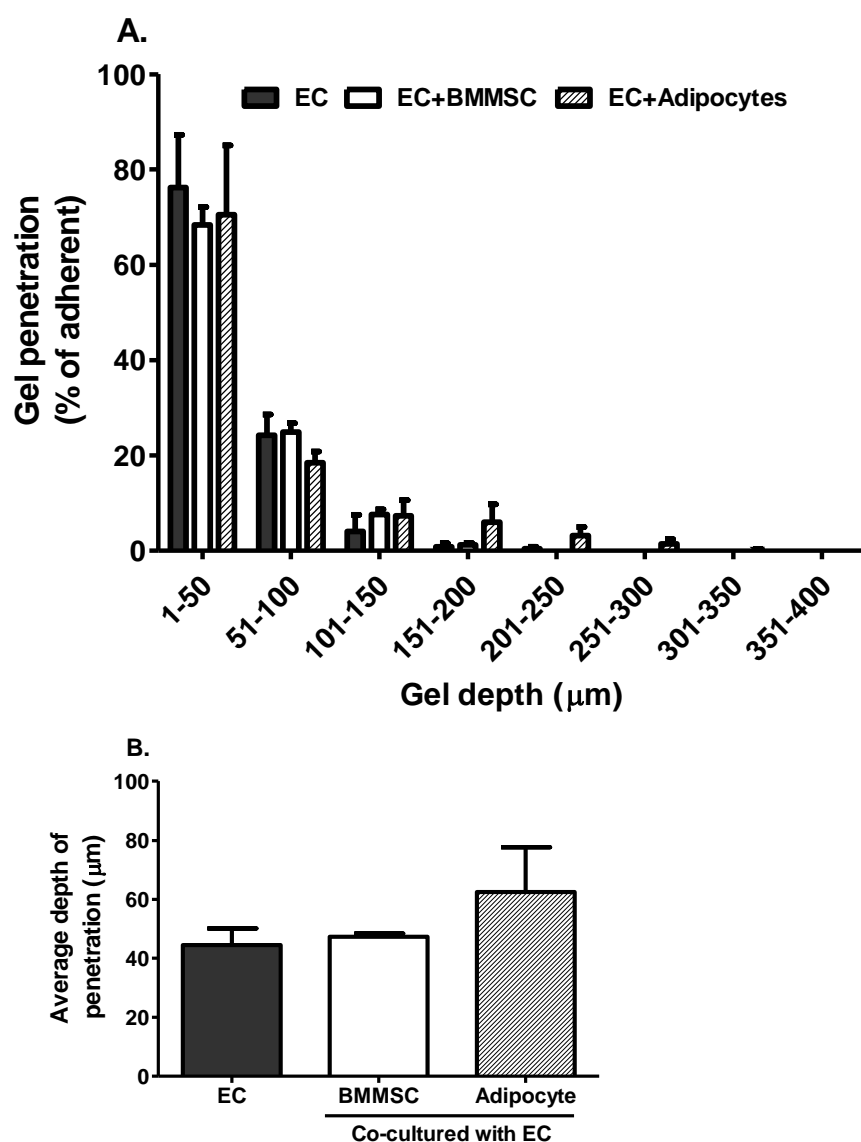


**Figure 5-21: Effect of adipocyte co-culture on the average depth of neutrophil penetration into 3D collagen gels in response to TNF $\alpha$ .** BMMSC or adipocytes were incorporated into collagen gels for 24h. EC were then seeded onto the gels for a further 24hr and were stimulated with 100U/ml TNF $\alpha$  for 4hr. Neutrophils ( $5 \times 10^5$ ) were added to gels for 20min and then non-adherent neutrophils were washed off. The average depth to which a neutrophil that penetrated into the gel migrated, was analysed after (A) 20min and (B) 120min. Data are mean  $\pm$  SEM from  $n = 3$  experiments. ANOVA showed no significant effect of culture conditions on the depth of neutrophil penetration. A different EC, neutrophil and BMMSC donor was used per experiment.



**Figure 5-22: Effect of BMMSC and BMMSC-derived adipocytes on neutrophil adhesion and migration into 3D collagen gels in the absence of cytokine.** BMMSC or adipocytes were incorporated into collagen gels for 24h. EC were then seeded onto the gels for a further 24hr.  $5 \times 10^5$  neutrophils were added to gels for 20min, non-adherent cells were washed off. Neutrophil adhesion and migration was assessed after 20min. (A) The percentage of neutrophils that were added to the gels that adhered after 20min. The percentage of adherent neutrophils that were (B) surface adherent, (C) transendothelial migrated and (D) penetrated into the gel were assessed. Data are mean  $\pm$  SEM from  $n = 3$  experiments. ANOVA showed no significant effect of culture conditions on neutrophil adhesion and migration through gels. A different EC, neutrophil and BMMSC donor was used per experiment.

Of those adherent cells, 20% were surface adherent and less than 6% were phase dark under the endothelium after 20min (Figure 5-22B and C). As with the cytokine-treated cultures (described above), 80% of adherent neutrophils penetrated into the gel but the majority were found close to the gel surface (less than 50µm deep) (Figure 5-22D and 5-23). The behaviour of adherent neutrophils was unaffected by adipocyte co-culture (Figure 5-22 and 5-23). Thus, adipocyte co-culture did not significantly promote the adhesion or migration of neutrophils in the absence of cytokines.



**Figure 5-23: Effect of adipocytes co-culture on the position and average depth of migrated neutrophils in 3D collagen gels in the absence of cytokine.** BMMSC or adipocytes were incorporated into collagen gels for 24h. EC were then seeded onto the gels for a further 24h and were stimulated with 100U/ml TNF $\alpha$  for 4hr. Neutrophils ( $5 \times 10^5$ ) were added to gels for 20min and then non-adherent neutrophils were washed off. (A) The position of neutrophils that penetrated into the gel were analysed by taking z-stack images in 2 $\mu$ m intervals through the gel. (B) The average depth of neutrophil penetration after 20min were analysed. Data are mean  $\pm$  SEM from  $n=3$  independent experiments. ANOVA showed no significant effect of culture conditions on neutrophil migration through gels and the average depth of neutrophil penetration. A different EC, neutrophil and BMMSC donor was used per experiment.

### 5.3. Discussion

In this chapter we aimed to assess the effects of differentiating MSC on their capacity to regulate leukocyte recruitment. We observed that after MSC from all 3 sources were differentiated into adipocytes, they had lost their ability to suppress neutrophil adhesion to cytokine-stimulated EC. Indeed, adipocyte co-culture tended to prime EC, so that when treated with a low dose TNF $\alpha$  (0.1U/ml or 1U/ml instead of 100U/ml), neutrophil recruitment was increased. In contrast, PBL adhesion remained suppressed by co-culture of EC with BMMSC-derived adipocytes. For those leukocytes that did adhere, adipocyte co-culture did not alter their capacity to migrate through endothelium and into matrix beneath with both EC types tested (HUVEC and BEC). IL-6 levels were as high in conditioned media from BMMSC-derived adipocyte mono-cultures and co-cultures as the levels for MSC co-cultures. Therefore, the loss of MSC-mediated suppression of neutrophil recruitment after adipogenic differentiation was not due to a reduction in IL-6 secretion. A reduction in IL-6R and IL-6ST gene expression was observed in adipocytes from co-culture compared to MSC from co-culture. However, we also observed that neutralisation of IL-6 restored the ability of MSC-derived adipocytes to suppress neutrophil adhesion. This suggested that rather than a reduction in the sensitivity of adipocyte co-cultures to IL-6, there was a shift in the action of IL-6 from being anti-inflammatory in MSC co-cultures to pro-inflammatory in MSC-derived adipocyte co-cultures. The suppression of neutrophil adhesion in the absence of IL-6 from adipocyte co-cultures, was likely mediated by other soluble factor(s) released. TGF $\beta$  was found in the previous chapter to play a role in mediating suppression of neutrophil recruitment (Section 4.2.6.). Here, expression of each of the three isoforms of TGF $\beta$  by EC at the gene level was significantly lower in adipocyte co-cultures than MSC co-cultures. Furthermore, we observed that neutralisation of TGF $\beta$



had no effect on neutrophil recruitment to MSC-derived adipocytes co-cultures, indicating that TGF $\beta$  did not play a role in mediating the effects of the differentiated adipocytes. Thus, in MSC co-culture there was complex interplay between IL-6 and TGF $\beta$  to downregulate neutrophil recruitment which was lost upon differentiation. To our knowledge, this is the first report to have examined the effects of adipogenic differentiation on the immunosuppressive effects of MSC. We have shown for the first time that adipogenic differentiation causes MSC to lose their ability to suppress neutrophil recruitment to inflamed EC.

In other models, BMMSC and ADMSC-derived osteoblasts and chondrocytes were able to suppress T-cell proliferation to the same extent as undifferentiated MSC (Montespan *et al.*, 2014; Niemeyer *et al.*, 2007). In contrast, we found that osteogenic differentiation of MSC reduced their capacity to suppress neutrophil adhesion to EC cultured on a collagen matrix. This suggests that while differentiation down the adipogenic or osteogenic lineages causes MSC to lose their capacity to suppress neutrophil recruitment, other immunosuppressive effects such as the inhibition of T-cell proliferation may persist. However, as the effect of MSC-derived adipocytes on T-cell proliferation has not yet been examined, we cannot say definitively that the suppression of neutrophil recruitment is the only immunosuppressive effect of MSC that is lost after differentiation.

Interestingly, we observed that BMMSC retained their capacity to suppress PBL recruitment after adipogenic differentiation. The mechanism(s) responsible for the suppression of PBL recruitment in this context were not identified and will require

further investigation. In addition, whether adipocytes utilise the same mechanism to suppress PBL recruitment as MSC in co-culture will also need to be evaluated. The capacity of MSC or adipocytes to mediate suppression of PBL recruitment may be due to a reduction in chemokine presentation on EC. A previous report showed that co-culture of fibroblasts in close contact with EC *in vitro* suppressed PBL, but not neutrophil, adhesion from flow (McGettrick *et al.*, 2010b). The suppression of PBL recruitment was not due to a reduction in adhesion molecules expression on EC in co-culture (McGettrick *et al.*, 2010b). Rather, fibroblasts reduced chemokine presentation (essential for stabilising PBL adhesion) by EC in response to cytokine-stimulation by secreting matrix metalloproteinases that likely cleaved key proteins involved in chemokine presentation by EC (McGettrick *et al.*, 2010b). It is possible that MSC and adipocytes mediated suppression of PBL recruitment through a similar mechanism. The effect of co-culture with MSC and MSC-derived adipocytes on chemokine presentation is therefore worthy of examination.

As noted above, the loss of immunosuppression by MSC after differentiation was not due to a reduction in IL-6 secretion. Conditioned media from adipocyte mono-cultures or co-cultures contained high levels of IL-6, but had no effect on neutrophil recruitment when added to EC. Thus, while IL-6 was present in the culture supernatant, it did not exert the same anti-inflammatory effects as it did in MSC co-culture. Expression of the components of the IL-6 receptor complex, IL-6R and IL-6ST, was reduced in adipocytes during co-culture compared to mono-culture. In contrast, expression of these genes was upregulated by MSC during co-culture compared to mono-culture. This suggests that adipocytes in co-culture likely had reduced sensitivity to IL-6 present in the conditioned medium. However, we found that the levels of sIL-6R protein were

the same in MSC and adipocyte co-culture conditioned media, suggesting that this mediator of the effects of IL-6 in MSC co-culture [Section 4.2.6. and (Luu *et al.*, 2013)] was not lacking in adipocyte co-culture. However, we observed that neutralisation of IL-6 in BMMSC-derived adipocyte co-cultures restored suppression of neutrophil recruitment. Therefore, the loss of suppression of neutrophil recruitment by MSC after differentiation was not likely to be due to a decrease in IL-6 sensitivity but instead due to a shift in the action of IL-6 from being anti-inflammatory (in MSC co-cultures) to pro-inflammatory (in adipocyte co-cultures).

IL-6 and TGF $\beta$  were both required to mediate the suppressive effects of MSC on neutrophil recruitment (Section 4.2.6.). Therefore, we hypothesised that the loss of suppression by MSC-derived adipocytes was due to a reduction in TGF $\beta$  expression during co-culture. However, as previously discussed (Section 4.3.), the levels of active TGF $\beta$  could not be measured in culture conditioned media. Therefore, we analysed expression of all TGF $\beta$  isoforms at the gene level. We observed that expression of TGF $\beta$  and its binding partner decorin, were reduced in EC co-cultured with adipocytes, compared to EC co-cultured with MSC. Expression of the three TGF $\beta$  isoforms were reduced in both MSC and adipocytes in co-culture. Reduced expression of TGF $\beta$  by EC may have contributed to the loss of suppression of neutrophil recruitment. Interestingly, a screen of cytokines and receptors found in MSC and adipocyte co-culture conditioned media suggested that more CD105 (an auxiliary TGF $\beta$  co-receptor) was present in conditioned medium from adipocyte co-culture compared to MSC co-culture. However, more detailed quantification of CD105 by ELISA showed that the levels of CD105 shed by the cells were similar in MSC and adipocyte co-culture conditioned media. Therefore, the loss of the immunosuppressive effect was not

attributable to a decrease in TGF $\beta$  signalling through shedding of CD105 in EC or adipocytes in co-culture. Nevertheless, we found that neutralisation of TGF $\beta$  had no effect on neutrophil recruitment to adipocyte co-cultures, suggesting that TGF $\beta$  had lost any activity in adipocyte co-cultures. Collectively, these data suggest that the loss of MSC-mediated suppression of neutrophil recruitment upon adipogenic differentiation was due to a change in the action of IL-6, possibly linked to loss of action of TGF $\beta$ .

Based on these observations, we suggest that suppression of neutrophil adhesion is lost upon differentiation of MSC through one or more of the following mechanisms:

1. The expression of a component of the IL-6 signalling pathway that mediates its anti-inflammatory effects is down-regulated or inhibited in adipocyte co-cultures.
2. A factor is generated that transforms the effect of IL-6 to make it pro-inflammatory.
3. IL-6 acts independently of TGF $\beta$  in adipocyte co-cultures and as a result the anti-inflammatory is lost.

The filter-based and collagen gel based models were used in this chapter to assess the effects of adipocytes on different stages of the recruitment cascade. The filter assay was used to assess neutrophil recruitment under physiological flow rates. The collagen gel assay was used to mimic the effects of adipocyte co-culture on the migration of neutrophils into tissue. The advantage of the gel model was that it enabled the migration of neutrophils through the EC and the underlying stroma to be analysed

in the same manner as would occur in tissue. However, the early stages of recruitment were not well represented in this model. Nevertheless, in the collagen gel model, we observed that differentiation of MSC into adipocytes or osteoblasts reversed the suppressive effects of MSC on neutrophil adhesion, as was the case in the flow-based adhesion model. In both models the migration of adherent neutrophils through the EC monolayer was unaffected by MSC or adipocyte co-culture with EC. In the matrix model specifically, migration (transendothelial migration, gel penetration and the depth of penetration) of neutrophils into the gel was unaffected by MSC or adipocyte co-culture. Thus, MSC exerted their effects on EC to limit neutrophil recruitment but did not act on EC to modulate transmigration or directly on neutrophils to alter their migration within the matrix'. Upon differentiation, adipocytes had no effect on any stage of the process.

The MSC and adipocytes isolated for this study were taken from healthy individuals. Previous studies have shown that excess OA (a monounsaturated free fatty acid) in the serum is taken up and metabolised by hepatocytes and converted to lipid contributing to the pathogenesis of non-alcoholic steatohepatitis (Ricchi *et al.*, 2009; Karim, 2013). To assess whether exposure of MSC-derived adipocytes to free fatty acids (associated with metabolic disorders) altered their effects on leukocyte recruitment, we treated adipocytes with OA for 24h prior to co-culture with EC. OA treatment inhibited the suppressive effects of MSC-derived adipocytes on PBL, but did not alter neutrophil adhesion. The mechanism by which OA caused loss of suppression of PBL adhesion was not explored and will require further investigation. The effect of OA treatment on MSC-derived adipocyte function may have physiological implications, as during chronic low grade inflammation, cytokines such as TNF $\alpha$  reduce the capacity

of adipose tissue to uptake and store lipid (Goossens, 2008; Sevastianova *et al.*, 2008; Salles *et al.*, 2012) which increases the levels of circulating fatty acids (such as OA). These free fatty acids may be taken up and stored by BMMSC-derived adipocytes and could adversely affect their ability to suppress PBL infiltration into inflamed tissue. In the future it would be interesting to test whether uptake of free fatty acids by undifferentiated MSC affects their adipogenic differentiation or alters their capacity to suppress leukocyte recruitment.

A question arising from our observations was whether adipose tissue derived AD exerted similar effects on recruitment as BMMSC-derived adipocytes. We observed that pre-AD and mature AD isolated from healthy subcutaneous adipose tissues were both able to suppress neutrophil and PBL adhesion to the same extent as BMMSC when co-cultured with EC. Similarly, co-culture with adipogenically differentiated pre-AD induced suppression of neutrophil and PBL recruitment to a similar extent as undifferentiated pre-AD. Thus, AD much like other stromal cell populations isolated from healthy individuals (McGettrick *et al.*, 2010b; Kuravi *et al.*, 2014; McGettrick *et al.*, 2009; Luu *et al.*, 2013), act as endogenous tissue-resident regulators, limiting the recruitment of leukocytes in response to pro-inflammatory cytokines. Interestingly, levels of IL-6 were high in co-culture media conditioned by pre-AD (undifferentiated and adipogenically differentiated) and mature AD. Thus these forms of AD appeared to retain anti-inflammatory effects of IL-6, in contrast to the BMMSC-derived adipocytes. Collectively, these data suggest that MSC-derived adipocytes are not the same as AD derived for adipose tissue, or AD differentiated from pre-AD from adipose tissue. It is reasonable to suggest that adipogenic differentiation of MSC in non-adipose tissue should not occur naturally, and that it may aberrantly decrease their

immunosuppressive capabilities in a way that does not occur in 'true' adipose tissue. Thus the lack of suppression by MSC-derived adipocytes observed in this chapter may resemble the situation in ectopic fat in chronically inflammatory conditions such as atherosclerosis (Liu *et al.*, 2014). To explore this further, the effects of adipocytes derived from ectopic fat from sites of chronic inflammation on leukocyte recruitment should be analysed, to assess whether they behave similarly to MSC-derived adipocytes. In addition, it would be interesting to test the effects of human osteoblasts and ectopic bone on leukocyte recruitment to determine whether the loss of suppression of neutrophil recruitment by MSC-derived osteoblasts mimics the behaviour of osteoblasts from healthy or chronically inflamed tissue. Results might imply that *in vitro* differentiation of MSC using current methods generates cells that do not represent those found in native fat or bone, at least in their effects on the immune system. Perhaps more interestingly, it might suggest that ectopic differentiation in chronic inflammation leads to an abnormal tissue unable to regulate leukocyte recruitment.

Due to the difficulty in obtaining tissue samples to prepare pre-AD and mature AD, the mechanism by which they mediated the suppression of neutrophil and PBL recruitment could not be investigated in the time available. In future, the roles of IL-6 and TGF $\beta$  in these co-cultures could be assessed to test whether they resemble their roles in suppression by MSC.

In conclusion, we have shown that MSC lose the capacity to regulate neutrophil, but not PBL, adhesion upon adipogenic differentiation using two different *in vitro* models.

Loss of suppression of neutrophil recruitment by MSC-derived adipocytes may be due to a switch in the mode of action of IL-6 from anti-inflammatory to pro-inflammatory, and loss of effect of TGF $\beta$ . Interestingly, adipocytes derived from MSC from all sources did not mimic adipose tissue-derived pre-AD and mature AD which were found to suppress both neutrophil and PBL recruitment. We suggest that aberrant adipogenic differentiation of tissue-resident MSC could 'permit' chronic inflammation by removing a constitutive regulation of infiltration of circulating leukocytes.



## **6. General discussion**

## 6.1. Summary of main findings

This thesis has examined the effects of MSC on the ability of EC to support leukocyte recruitment, a key process in inflammation. In particular, we focused on whether MSC from different sources (bone marrow, Wharton's jelly and trabecular bone) and adipose tissue-derived stromal cells are capable of modulating recruitment to inflamed vascular EC. The effect of adipogenic differentiation of MSC and adipose tissue-derived pre-AD on their regulatory capacity was also explored. To examine recruitment, two *in vitro* flow based adhesion assay models were used; a microslide channel model mimicking the effects of systemically infused MSC and a filter-based model mimicking the effects of direct injection of MSC into the tissue. A 3D collagen gel based static migration system was used to analyse the effect of MSC, adipocyte or osteoblasts on the migration of neutrophils. The main findings of this thesis are:

1. MSC from different tissues have a similar capacity to suppress neutrophil adhesion to TNF $\alpha$ -stimulated EC. The effect was diminished albeit to varying degrees in the absence of contact and with MSC passage.
2. The suppressive effect of BMMSC and WJMSC on neutrophil adhesion was mediated, at least in part, by IL-6 and TGF $\beta$ .
3. In contrast to differentiated pre-AD and mature AD, differentiation of MSC into adipocytes abrogated their suppressive effects on neutrophil, but not PBL, recruitment.
4. IL-6 was identified as a key factor mediating the loss of suppression after adipogenic differentiation indicating context-specific differences in its action on target cells, to regulate recruitment.

## 6.2. Significance of these findings

It has previously been shown that direct co-culture of BMMSC with EC in microslide channels suppressed neutrophil as well as PBL adhesion and transmigration (Luu *et al.*, 2013). Here, it was observed that MSC from different sources shared a similar capacity to suppress neutrophil adhesion, but did not affect transmigration. This correlates with previous reports that other tissue-resident stromal cells such as dermal fibroblasts and podocytes are also capable of suppressing leukocyte recruitment to vascular EC (Kuravi *et al.*, 2014; McGettrick *et al.*, 2009). In all cases, IL-6 signalling was involved in mediating suppression, although the effects on recruitment varied depending on the stromal cells in co-culture. For instance, dermal fibroblast-mediated suppression of PBL adhesion was reduced by neutralising IL-6 or TGF $\beta$ , where complete reversal of the suppression on PBL adhesion only occurred when both factors were neutralised (McGettrick *et al.*, 2009). In contrast, we showed here that WJMSC and BMMSC required both IL-6 and TGF $\beta$  to mediate their suppressive effects as neutralisation of either abrogated the response, with one factor likely acting upstream of the other. In podocyte co-cultures, IL-6 alone was sufficient to drive suppression of neutrophil recruitment and its neutralisation was found to completely abrogate the effect (Kuravi *et al.*, 2014). Therefore, the actions of IL-6 and TGF $\beta$  are context dependent, and vary depending on the stromal cell type in co-culture with EC. Importantly, the exact nature of the interactions between IL-6 and TGF $\beta$  during co-culture is not well understood. Given that MSC from different sources have the same immune-protective effects, MSC alongside other tissue-resident stromal cells may act in concert to suppress leukocyte recruitment in response to inflammatory cytokines.

While MSC from different tissue sources exert similar immunosuppressive effects (Roemeling-van Rhijn *et al.*, 2013; Jin *et al.*, 2013; Puissant *et al.*, 2005), here it was found that the capacity of BMMSC, and to a much lesser extent WJMSC, to suppress neutrophil recruitment was diminished with passage. Previous studies have also reported that foetal and neonatal MSC populations such as foetal liver-derived MSC retain their capacity to suppress T-cell proliferation at a much higher passage than BMMSC (Giuliani *et al.*, 2011; Yu *et al.*, 2014; Zhuang *et al.*, 2014). This suggests that foetal and neonatal MSC retain their immunosuppressive effects for longer than BMMSC after prolonged culture. However, to our knowledge there are currently no studies that have directly compared the effects of passage on the immunomodulatory effects of WJMSC and BMMSC. Furthermore, the few studies that have examined the effect of passage on MSC immune function, T-cell proliferation and activation were the only parameters measured. Whether other immunosuppressive effects of MSC are sensitive to passage remains to be explored. For the first time it has been shown that in the same model, using the same culture conditions, BMMSC lose their immunomodulatory capacity with passage, as assessed by measuring neutrophil recruitment at an earlier passage than WJMSC. This has important therapeutic implications as it suggests that culture expansion of BMMSC to generate sufficient numbers to treat patients with chronic inflammatory conditions may reduce their efficacy in suppressing leukocyte infiltration into the inflamed site. Therefore, WJMSC could potentially be a better therapeutic intervention in this context.

Importantly, the suppressive effects of all MSC populations on recruitment were shown to be mediated by soluble agents (including IL-6) which were only generated during crosstalk between EC and MSC, as mono-culture conditioned medium from MSC had

no effect on recruitment. Crosstalk between BMMSC and EC could be achieved at a much greater distance (~900µm) than WJMSC and TBMSC, which required close proximity with EC to exert their effect. This has important therapeutic implications as studies have shown that systemically infused MSC have a low homing efficiency (<1%) and tend to become lodged in the lungs where they mediate their effects through the release of soluble factors (Eggenhofer *et al.*, 2014; Le Blanc and Mougiakakos, 2012; Eggenhofer *et al.*, 2012; Kavanagh *et al.*, 2015). Mechanical trapping of MSC in lung vasculature would enable systemically infused MSC to come into contact with EC and mediate crosstalk. However, as systemically administered MSC are short lived and often migrate to different sites over time [reviewed by (Eggenhofer *et al.*, 2012; Le Blanc and Mougiakakos, 2012), the duration of direct contact may be insufficient to mediate the crosstalk required to suppress recruitment. However, as BMMSC retain their ability to crosstalk with EC at a distance, systemically infused BMMSC may have beneficial effects over greater distances even in the absence of contact with EC. In contrast, WJMSC and TBMSC required close proximity with vascular EC to exert their immunosuppressive effects on recruitment. Therefore, direct injection of WJMSC and TBMSC into the inflamed tissue would be more likely to promote crosstalk with EC, as tissue-resident stromal cells are usually in close proximity to the vasculature. As BMMSC could mediate their suppressive effects in the presence or absence of contact, either route of administration could be used to deliver MSC for treatment of chronic inflammatory diseases. Clinicians must consider the effects of MSC passage and requirement for proximity with target cells when selecting the optimum MSC for therapeutic use, tailoring the treatment to the condition.

While it has recently been shown that the inflammatory microenvironment may transform MSC and modify their immunosuppressive capabilities (Prasanna *et al.*, 2010; Waterman *et al.*, 2010), the potential effects of MSC differentiation on their suppressive capabilities has not been widely explored. Here, it was observed that adipogenic differentiation of MSC from different sources diminished their suppressive capacity to suppress neutrophil recruitment in flow and gel based static adhesion models. Previous reports have only shown that osteogenic and chondrogenic differentiation did not significantly alter the immunosuppressive function of MSC (Niemeyer *et al.*, 2007; Montespan *et al.*, 2014; Lohan *et al.*, 2014), although T-cell proliferation was the only parameter measured. In a conflicting report, rat MSC-derived chondrocytes lost the ability to suppress allogeneic T-cell proliferation *in vitro*. This was thought to be due to a reduction in nitric oxide and PGE<sub>2</sub> secretion (Ryan *et al.*, 2014). To our knowledge, the abovementioned studies are the only others where the effect of differentiation on MSC immunomodulation have been examined. Conflicting findings within these studies may be due to differences in the MSC source (rat vs. human MSC) used or the method used to isolate and differentiate the MSC. However, the effects of adipogenic differentiation on T-cell proliferation has not been previously analysed.

It was also observed that adipogenic differentiation of MSC alters their crosstalk with EC, reducing their ability to suppress neutrophil recruitment. IL-6 was a key mediator in the loss of immunosuppression by MSC-derived adipocytes, as neutralisation of IL-6 restored their capacity to limit neutrophil recruitment. Thus, IL-6 acts as a context-dependent cytokine that promotes suppression of neutrophil adhesion by MSC but drives recruitment upon MSC differentiation into adipocytes. These data suggest that aberrant adipogenic differentiation of MSC could be pathological as MSC no longer act

to dampen the inflammatory infiltrate but rather have the capacity to contribute to aberrant inflammation through crosstalk with EC or immune cells, adversely affecting their effector functions. MSC may be contributors of ectopic fat formation as cells that possess MSC features were found to accumulate lipid in response to tissue damage (Sohn *et al.*, 2013, 2015; Uezumi *et al.*, 2010). Furthermore, depletion of these cells prevented deposition of ectopic fat in response to tissue damage (Uezumi *et al.*, 2010). As ectopic fat is likely caused by the aberrant accumulation of lipid in tissue-resident cells in non-adipose tissues (Gustafson and Smith, 2006; Filippin-Monteiro *et al.*, 2012; Sevastianova *et al.*, 2008; Salles *et al.*, 2012), it is possible that in the inflammatory environment, MSC may undergo adipogenic differentiation taking up excess lipid in the local milieu.

Interestingly, it was also observed that MSC-derived adipocytes do not have the same effects on recruitment as healthy adipose tissue-derived pre-AD and mature AD, which were able to suppress neutrophil and PBL adhesion to the same extent as undifferentiated MSC. This suggests that healthy adipose tissue-derived stromal cells, much like other tissue-resident stromal populations (i.e. MSC, dermal fibroblasts and podocytes) are inherently immunosuppressive and act to dampen leukocyte recruitment (Kuravi *et al.*, 2014; McGettrick *et al.*, 2009). Moreover, adipogenically differentiated pre-AD *in vitro* were also capable of suppressing recruitment to a similar extent as the undifferentiated cells. This suggests that unlike the other tissue-derived MSC tested (BMMSC, WJMSC and TBMSC), adipose tissue-derived stromal cells uniquely retain their suppressive effects upon differentiation. This may be a tissue-specific function of pre-AD and mature AD to limit inappropriate recruitment of leukocytes into healthy adipose tissue. It also suggests that upon differentiation MSC

lose their immunosuppressive capabilities and adopt a more stimulatory phenotype, potentially pro-inflammatory phenotype.

### **6.3. Conclusion**

In conclusion, tissue-resident MSC from different sources were found to undergo crosstalk with vascular EC to limit neutrophil recruitment in response to inflammatory cytokines to a similar degree. IL-6 and TGF $\beta$  were identified as common bioactive agents in all co-cultures and a requirement for close proximity between EC and MSC was also observed. Differences in their expansion potential and requirement for proximity with EC indicates that there are functional differences between MSC from different sources. Other stromal populations from healthy tissues also exert suppressive effects on leukocyte recruitment (Kuravi *et al.*, 2014; Luu *et al.*, 2013), while stromal cells from chronically inflamed sites may enhance the inflammatory influx (Lally *et al.*, 2005; Luu *et al.*, 2013; Kuravi *et al.*, 2014; McGettrick *et al.*, 2009). In addition, it was observed that differentiation of MSC, but not pre-AD, into adipocytes reduced their suppressive effects on neutrophil recruitment in an IL-6 dependent manner. As such, differentiation of MSC *in vivo* could be pathogenic in chronic inflammation, as it would enhance the infiltration of circulating neutrophils. Thus, MSC from different tissues share the endogenous capacity to regulate EC and the inflammatory infiltrate, akin to other healthy stromal cells.

Triggering MSC adipogenic differentiation in chronically inflamed or damaged tissue may contribute to the pathology of the disease by enabling the aberrant influx of circulating neutrophils. In terms of therapy, there are advantages and disadvantages



to using each of the MSC populations. The limited expansion potential of TBMSC makes it an undesirable source for therapy. While WJMSC exhibit a greater expansion potential, the requirement for close proximity with EC to mediate their effects may limit their use. Furthermore, while BMMSC are able to undergo crosstalk with EC over a greater distance, loss of their immunosuppressive effects upon expansion also limits their use therapeutically.

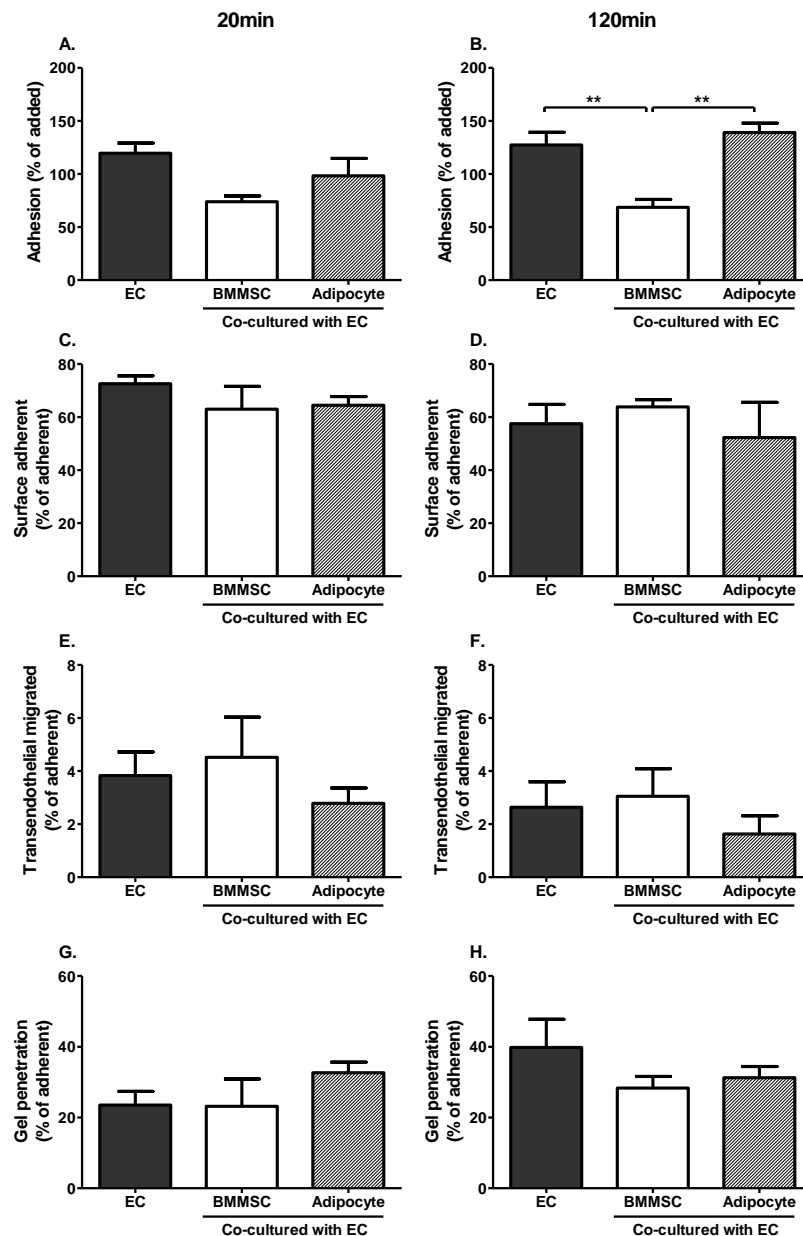
#### **6.4. Future work**

The data shown in this thesis raises further questions such as:

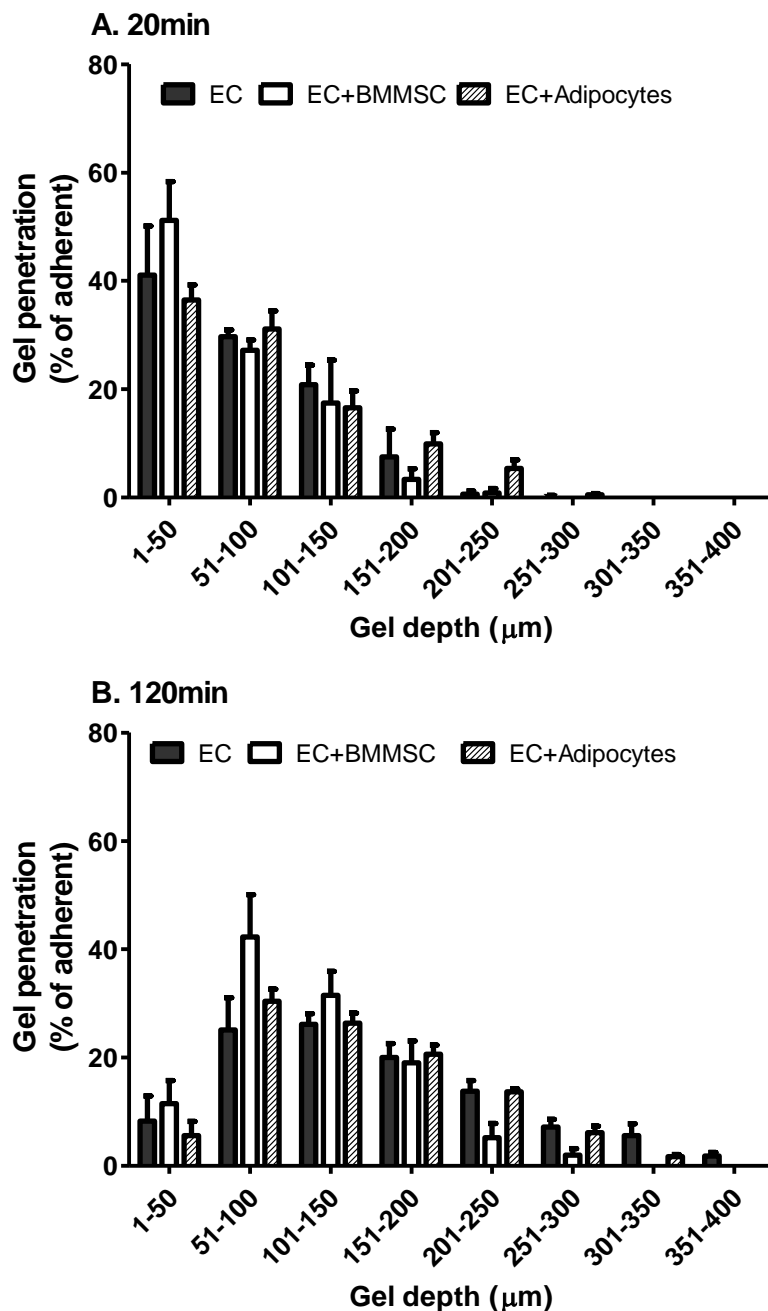
1. How do soluble mediators generated during crosstalk between EC and MSC exert their effects on leukocyte recruitment? To address this question, the following should be examined:
  - a. The mechanism by which IL-6 and TGF $\beta$  signal during co-culture to mediate the suppressive effects; identifying which cell type in the co-culture responds to these factors, which factor signals first and how it regulates the activity of the other.
  - b. Identifying other bioactive mediator(s) that also play a role in suppression that are presumably lost in co-culture with later passage MSC or in co-cultures generated in the absence of contact.
  - c. Observing other phenotypic changes e.g. expression of adherens junctions in EC during co-culture that might be linked to the immunosuppressive effects observed.

2. What changes in bioactive mediator(s) are responsible for the loss of suppression of neutrophil recruitment upon adipogenic differentiation? To address this question, the following should be examined:
- a. Identifying factors regulating IL-6 signalling that are differentially active in MSC vs. MSC-derived adipocyte co-cultures that may shift its activity from anti-inflammatory to pro-inflammatory.
  - b. Identify factors that are present in MSC-derived adipocytes co-cultures that may suppress PBL adhesion (e.g. chemokine expression by EC).
  - c. Determining if the loss of suppression by MSC-derived adipocytes, but not differentiated pre-AD, is a culture induced phenomenon or due to tissue-specific differences between the two cell types.
  - d. Assessing the bioactivity of MSC and MSC-derived adipocytes co-culture conditioned media *in vivo*.
  - e. Determining whether ectopic fat observed in sites of chronic inflammation could be derived from aberrant differentiated MSC.

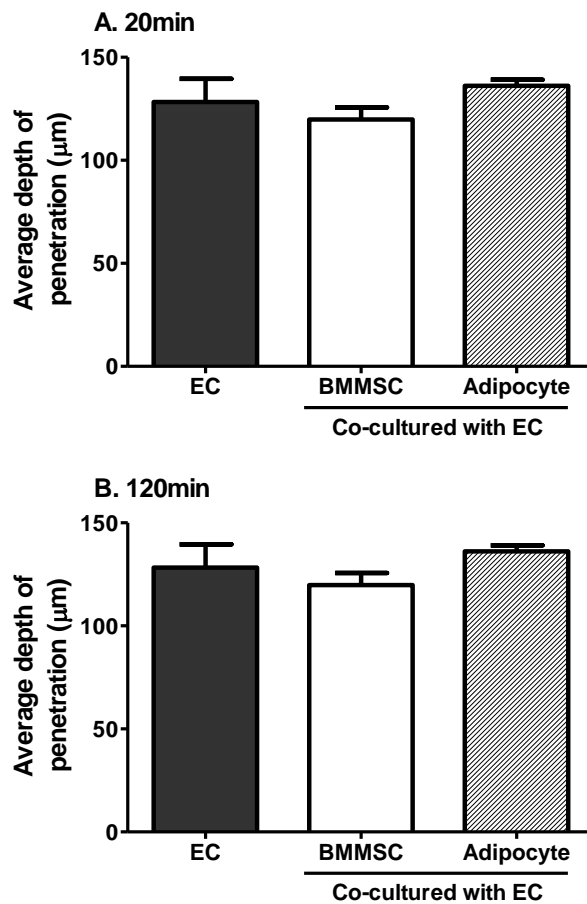
## **7. Appendix**



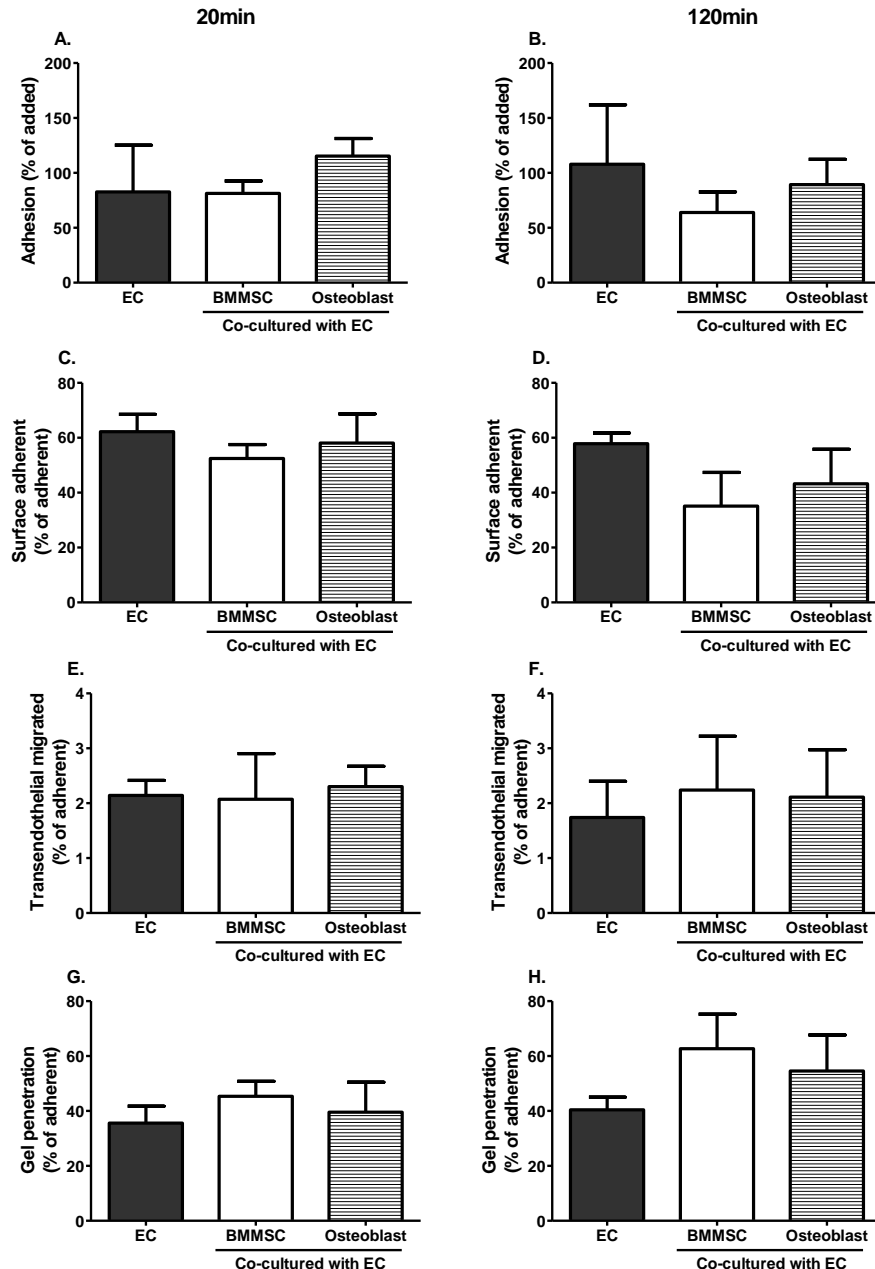
**Appendix 1-1: Effect of adipocyte differentiation on neutrophil adhesion to BEC and their migration into 3D collagen gels in response to TNF $\alpha$ .** BMMSC or adipocytes were incorporated into collagen gels for 24h. BEC were then seeded onto the gels for a further 24hr and were stimulated with 100U/ml TNF $\alpha$  for 4hr. Neutrophils ( $5 \times 10^5$ ) were added to gels for 20min, non-adherent cells were washed off. Neutrophil adhesion and migration was assessed after 20min and 120min. (A,B) The percentage of neutrophils that were added to the gels that adhered after 20min and 120min respectively. The percentage of adherent neutrophils that were (C,D) surface adherent, (E,F) transendothelial migrated and (G,H) penetrated into the gel were assessed. Data are mean  $\pm$  SEM from  $n = 3$  experiments. In B, ANOVA showed a significant effect of culture conditions on neutrophil adhesion,  $p < 0.01$ . \*\* $p < 0.01$  by Bonferroni post-test. In C-H, ANOVA showed no significant effect of culture conditions on neutrophil migration through gels. A different EC and neutrophil donor was used per experiment and 2 BMMSC donors were used in this series of experiments.



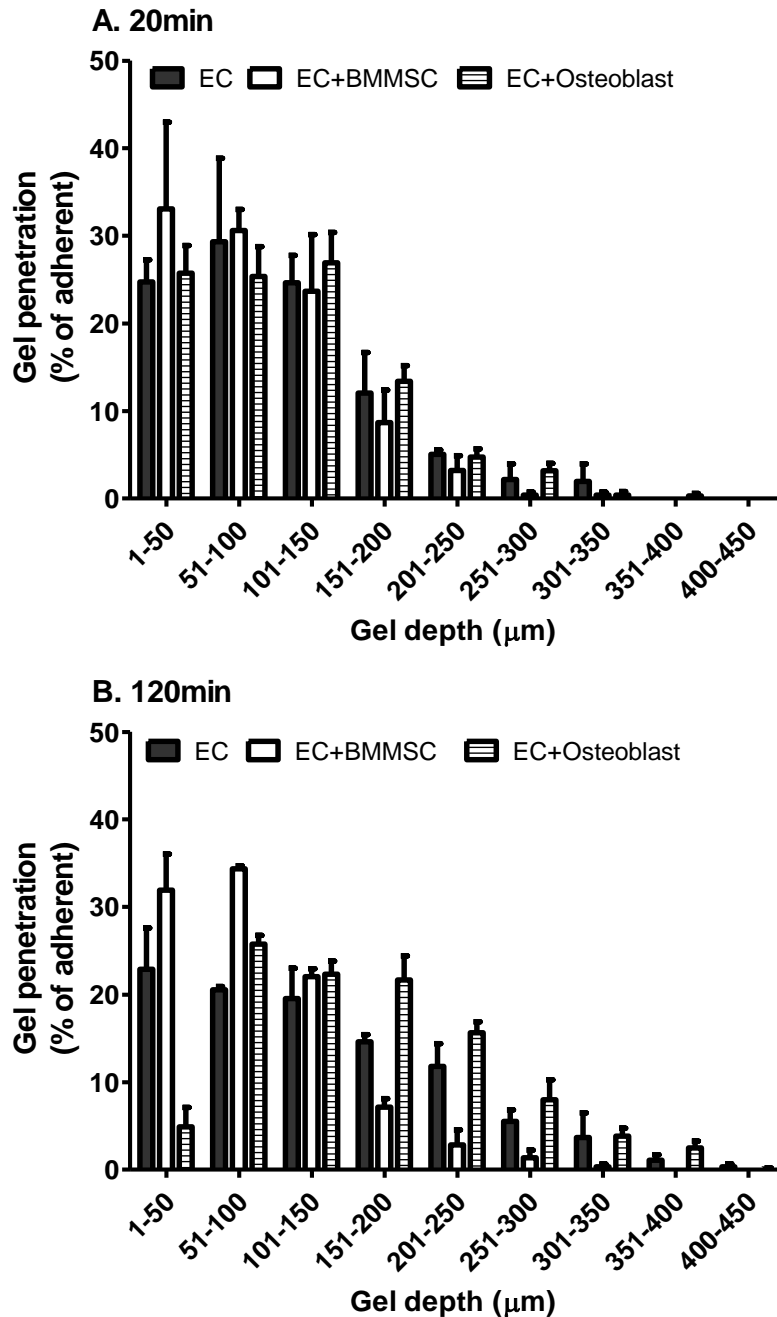
**Appendix 1-2: Effect of adipocytes co-culture on the position of migrated neutrophils within 3D collagen gels in response to TNF $\alpha$ .** BMMSC or adipocytes were incorporated into collagen gels for 24h. BEC were then seeded onto the gels for a further 24hr and were stimulated with 100U/ml TNF $\alpha$  for 4hr. Neutrophils ( $5 \times 10^5$ ) were added to gels for 20min and then non-adherent neutrophils were washed off. The position of neutrophils that penetrated into the gel were analysed by taking z-stack images in 2 $\mu$ m intervals through the gel after (A) 20min and (B) 120min. Data are mean  $\pm$  SEM from n= 3 experiments. ANOVA showed no significant effect of culture conditions on neutrophil migration through gels. A different EC and neutrophil donor was used per experiment and 2 BMMSC donors were used in this series of experiments.



**Appendix 1-3: Effect of adipocyte co-culture on the average depth of neutrophil penetration into 3D collagen gels in response to TNF $\alpha$ .** BMMSC or adipocytes were incorporated into collagen gels for 24h. BEC were then seeded onto the gels for a further 24hr and were stimulated with 100U/ml TNF $\alpha$  for 4hr. Neutrophils ( $5 \times 10^5$ ) were added to gels for 20min and then non-adherent neutrophils were washed off. The average depth to which a neutrophil that penetrated into the gel migrated, was analysed after (A) 20min and (B) 120min. Data are mean  $\pm$  SEM from n= 3 experiments. ANOVA showed no significant effect of culture conditions on the depth of neutrophil penetration through gels. A different EC and neutrophil donor was used per experiment and 2 BMMSC donors were used in this series of experiments.

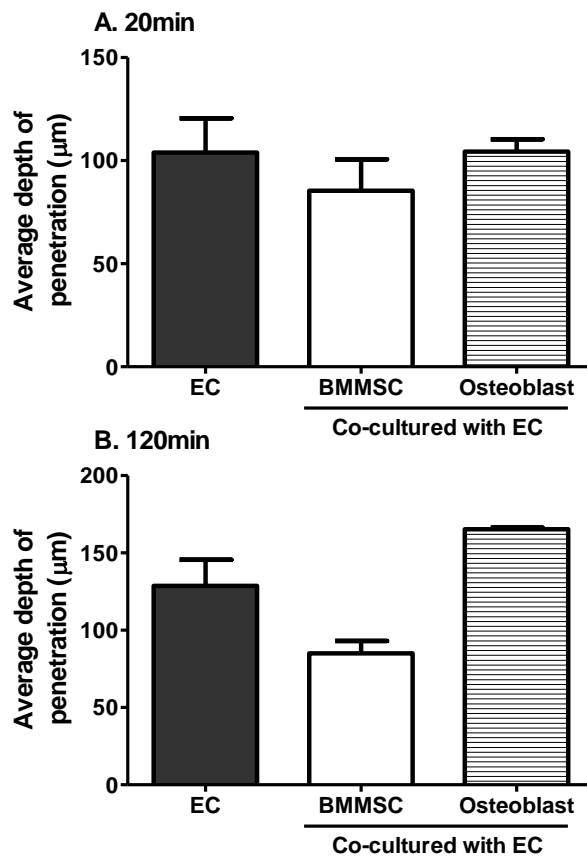


**Appendix 1-4: Effect of osteoblast differentiation on neutrophil adhesion to BEC and their migration into 3D collagen gels in response to TNF $\alpha$ .** BMMSC or osteoblasts were incorporated into collagen gels for 24h. BEC were then seeded onto the gels for a further 24hr and were stimulated with 100U/ml TNF $\alpha$  for 4hr. Neutrophils ( $5 \times 10^5$ ) were added to gels for 20min, non-adherent cells were then washed off. Neutrophil adhesion and migration was assessed after 20min and 120min. (A,B) The percentage of neutrophils that were added to the gels that adhered after 20min and 120min respectively. The percentage of adherent neutrophils that were (C,D) surface adherent, (E,F) transendothelial migrated and (G,H) penetrated into the gel were assessed. Data are mean  $\pm$  SEM from n= 3 experiments. ANOVA showed no significant effect of culture conditions on neutrophil adhesion and migration through gels. A different EC and neutrophil donor was used per experiment and 2 BMMSC donors were used in this series of experiments.



**Appendix 1-5: Effect of osteoblast co-culture on the position of migrated neutrophils within 3D collagen gels in response to TNF $\alpha$ .** BMMSC or osteoblasts were incorporated into collagen gels for 24h. BEC were then seeded onto the gels for a further 24hr and were stimulated with 100U/ml TNF $\alpha$  for 4hr. Neutrophils ( $5 \times 10^5$ ) were added to gels for 20min and then non-adherent neutrophils were washed off. The position of neutrophils that penetrated into the gel were analysed by taking z-stack images in 2μm intervals through the gel after (A) 20min and (B) 120min. Data are mean  $\pm$  SEM from n= 3 experiments. ANOVA showed no significant effect of culture conditions on neutrophil migration through gels. A different EC and neutrophil donor was used per experiment and 2 BMMSC donors were used in this series of experiments.





**Appendix 1-6: Effect of osteoblast co-culture on the average depth of neutrophil penetration into 3D collagen gels in response to  $\text{TNF}\alpha$ .** BMMSC or osteoblasts were incorporated into collagen gels for 24h. BEC were then seeded onto the gels for a further 24hr and were stimulated with 100U/ml  $\text{TNF}\alpha$  for 4hr. Neutrophils ( $5 \times 10^5$ ) were added to gels for 20min and then non-adherent neutrophils were washed off. The average depth to which a neutrophil that penetrated into the gel migrated, was analysed after (A) 20min and (B) 120min. Data are mean  $\pm$  SEM from  $n = 3$  experiments. ANOVA showed no significant effect of culture conditions on the depth of neutrophil penetration through gels. A different EC and neutrophil donor was used per experiment and 2 BMMSC donors were used in this series of experiments.

## **Publications arising from this thesis**

### *Published papers*

**H. Munir** and H.M. McGettrick. Mesenchymal stem cells therapy for autoimmune disease: risks and rewards. *Stem Cells and Development*. 24, 18, p2091-100, Sep 2015. Review

**H. Munir**, G.E. Rainger, G.B. Nash, and H.M. McGettrick (2015). Analysing the effects of stromal cells on the recruitment of leukocytes from flow. *Journal of Visualised Experiments*. 95, e52480.

### *Published abstracts*

**H. Munir**, H.M. McGettrick, and G.B. Nash. Adipogenic differentiation of human MSC reverses their immunosuppressive effects. (4th European Congress of Immunology, Vienna, Austria, September 2015)

L. Clarke, H. Munir, R. Andrews, G.B. Nash and H.M. McGettrick. Mesenchymal stem cells influence the ability of inflamed endothelial cells to recruit neutrophils: A comparative study. (4th European Congress of Immunology, Vienna, Austria, September 2015)

H.M. McGettrick, **H. Munir**, C.S. Davies, K. Raza, G.B. Nash, C.D. Buckley and A. Filer. A transitional fibroblast phenotype exists in very early rheumatoid arthritis: Modulation of endothelial responses and lymphocyte recruitment. (4th European Congress of Immunology, Vienna, Austria, September 2015)

**H. Munir**, H.M. McGettrick, and G.B. Nash Adipogenic differentiation of human MSC reverses their immunosuppressive effects. (International Society for Stem Cell Research, Stockholm, Sweden, June 2015)

A. Alanazi, **H. Munir**, H.M. McGettrick, S.P. Watson, G.B. Nash. Adhesion of mesenchymal stem cells from flowing blood: effects of their tissue origin and of interactions with platelets. (Biorehology Congress, Seoul, South Korea, June 2015)

H.M. McGettrick, C.D. Buckley, **H. Munir**, H.C. Jeffery, C.S. Davies, K. Raza, G.B. Nash and A. Filer. Identification of a transitional fibroblast phenotype in early rheumatoid arthritis. *Immunology*, 143s1:57 (British Society for Immunology Congress, Brighton, UK, December 2014)

**H. Munir**, N-T. Luu, H.M. McGettrick and G.B. Nash. Comparative ability of mesenchymal stem cells from different sources to regulate neutrophil recruitment by endothelial cells. (British Society for Immunology: Stromal Immunology Affinity Group, Cambridge, UK, November 2013)

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